THE
BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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THE BIOLOGICAL BULLETIN

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THE MARINE BIOLOGICAL LABORATORY

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R. RUGH, College of Physicians and Surgeons
H. B. STEINBACH, University of Minnesota

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TO SERVE UNTIL 1957

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II. ACT OF INCORPORATION

No. 3170

COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The members of the Corporation shall consist of persons elected by the Board of Trustees.

II. The officers of the Corporation shall consist of a President, Vice President, Director, Treasurer, and Clerk.

III. The Annual Meeting of the members shall be held on the Friday following the second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these By-laws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least fifteen (15) days before such meeting. to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held promptly after the Annual Meeting of the Corporation at the Laboratory in Woods Hole, Mass. Special meetings of the Trustees shall be called by the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-two Trustees chosen by the Corporation, divided into four classes, each to serve four years. After having served two consecutive terms of four years each. Trustees are ineligible for re-election until a year has elapsed. In addition, there shall be two groups of Trustees as follows:

(B) Trustees ex officio, who shall be the President and Vice President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer, and the Clerk:

(C) Trustees Emeriti, who shall be elected from *present* or *former* Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next Annual Meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee *Emeritus* for life. The Trustees *ex officio* and *Emeritus* shall have all the rights of the Trustees except that Trustees *Emeritus* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corporation; they shall elect a President of the Corporation who shall also be Chairman of the Board of Trustees and who shall be elected for a term of five years and shall serve until his successor is selected and qualified; and shall also elect a Vice President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and who shall be selected for a term of five years and shall serve until his successor is selected and qualified; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

XI. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

XII. The account of the Treasurer shall be audited annually by a certified public accountant.

XIII. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

IV. REPORT OF THE DIRECTOR

To the Trustees of the Marine Biological Laboratory:

Gentlemen:

I submit herewith the report of the sixty-ninth session of the Marine Biological Laboratory.

Since the close of World War II there have been certain developments at the Laboratory which have reduced the amount of research space available to summer investigators. It has been necessary to set aside some space formerly assigned to investigators for special instrumentation. Seven laboratories are now used for radiobiology, electron microscopy, dehumidified laboratories for special equipment and the personnel responsible for these services. Eight laboratories are used by investigators working on a year-round basis so are not available for the summer investigators. Thus space for about thirty summer investigators has been diverted to other uses.

Every effort has been made to accommodate as many summer investigators as possible which has probably produced serious crowding in some areas. The establishment of the Neuromuscular Training Program will further reduce the research space available for summer investigators. In recent years there has been a marked increase in the number of applicants for research space with many well qualified individuals being turned away every year. Serious consideration should be given to the expansion of our research facilities. At least the Laboratory should pick up the space lost to other activities. This can best be done by replacing the Rockefeller, Botany and Old Lecture Hall with a modern brick building. Additional housing should be constructed at the same time to avoid over-taxing our present housing and dining hall facilities.

1. Grants, Contracts and Contributions

The total income from these sources of support amounted to $204,034.00 in 1956. This represents 40% of the total income of the Laboratory and consists of the following accounts:

American Cancer Soc.—C26AS—Function of Nuclei and Nucleic Acids	$ 18,705.00
American Cancer Soc.—R-7F—Fundamental Studies in Radiobiology	6,600.00
A.E.C.—At 30-1-1343—Program of Research on the Physiology of Marine Organisms Using Radioisotopes	8,450.00
N.I.H.—B643C—Encephalization in Embryonic Development	2,012.00
N.I.H.—SA43PH 423—Investigations of the Microscopic Physiology of Various Forms of Living Marine Life	1,350.00
N.I.H.—B799—Electrical and Mechanical Changes in Muscle	920.00
N.I.H.—RG4359—Biological Research on the Morphology, Ecology, Physiology, Biochemistry and Biophysics of Marine Organisms	40,000.00
N.I.H.—RG-E-4513—Radiation-Induced Paralysis in Protozoa	14,950.00
National Science Found.—G2142—Funds for Biological Research	25,000.00
National Science Found.—G1807—Mechano-Chemical Coupling in Muscle	11,500.00
National Science Found.—G1395—Osmoregulation of Excretion in Tunicates	2,917.00
National Science Found.—G2655—Structure and Function of Proteins	7,750.00
O.N.R.—1497—Studies in Marine Biology	15,000.00
O.N.R.—09701—Studies on Isolated Nerve Fibers	6,450.00
O.N.R.—09702—Investigation of Environmental Factors Influencing Certain Marine Biological Populations in the Woods Hole area	5,000.00
American Philosophical Society	2,500.00
M. B. L. Associates	5,255.00
Eli Lilly Company	5,000.00
Rockefeller Foundation	20,000.00
Upjohn Company	2,000.00
Ciba Company	1,000.00
Grass Trust	1,000.00
Other	675.00
	$204,034.00

2. Neuromuscular Training Program

A neuromuscular training program has been set up under the supervision of Dr. Stephen Kuffler, which will have a staff of three investigators and eight post-doctoral Fellows. This is a multidisciplinary program which will be housed in special quarters developed in the Crane Building. The program is being supported by the Public Health Service.

3. Plant Improvements

During the past winter the Supply Department Building was completely reconstructed. Extensive changes were made in the internal arrangement of the building which adapt it much more satisfactorily to its various functions. Included in the building will be a study museum containing specimens of the various forms available in the Woods Hole area for research. Mr. Milton B. Gray, who is very well acquainted with the local fauna, will serve as Curator of the Museum.

The Crane Wing of the Brick Building was built in 1913. It has been in serious need of rehabilitation for the past several years. Many of the facilities were not adequate for much of the research currently in progress at the Laboratory. A request for a grant of $415,000 was made to the National Science Foundation to reconstruct and modernize this building. Early in this year, favorable action was taken by the National Science Foundation on this grant request. This reconstruction work will be undertaken between the 1957–1958 summer seasons. The resulting building should be adequate for any type of research undertaken at the Laboratory.

4. Instruction

In line with the Laboratory's policy Dr. Bostwick H. Ketchum will retire as head of the Marine Ecology course, having served a five-year term. He will be succeeded by Dr. Eugene P. Odum of the University of Georgia.

Dr. Stephen Kuffler has resigned as head of the Physiology course to take over the leadership of the Neuromuscular Training Program. He will be succeeded by Dr. W. D. McElroy of the Johns Hopkins University.

The course in Marine Ecology was originally established under Dr. Ketchum's direction who can take real pride in the way it has developed. Dr. Kuffler maintained the course in Physiology at the high level of effectiveness which has characterized it for many years.

5. Retirements

The Laboratory is losing the services of three of its permanent staff who have served the Laboratory most faithfully for many years. Mr. James McInnis is retiring as Manager of the Supply Department, having served in that department for thirty-eight years.

Miss Polly Crowell has been connected with the Laboratory administration for forty-one years and Miss Ruth Crowell with the Supply Department for thirty-seven years. The success of the Laboratory depends in a large measure on the effectiveness of its staff, of which they have been outstanding members.

6. Deaths

This past year the Corporation of the Marine Biological Laboratory suffered the loss of one of its most eminent and loyal members, in the death of Dr. B. M. Duggar. A memorial to Dr. Duggar will be presented at the Annual Meeting of 1957.

At the Annual Meeting of 1956 Dr. Mary Sears read a memorial for Mrs. Priscilla B. Montgomery. Mrs. Montgomery was for many years the Librarian of the Laboratory and over the years her efforts on behalf of the Library have made it what it is today. Her name will always be remembered with affection and pride by all who knew her.

Respectfully submitted,

PHILIP B. ARMSTRONG,
Director

MEMORIAL

Priscilla Braislin Montgomery

by

Mary Sears

28 December 1874–9 August 1956

In 1897, recent graduates of women's colleges came to Woods Hole for summer courses just as they do today. Priscilla Braislin of Crosswicks, New Jersey, arrived from Vassar and enrolled in the Embryology Course. The next year, Thomas Harrison Montgomery came as an investigator and in 1900 he became an instructor in the bird section of the nature study course. Priscilla Braislin was back to take this course after three years of teaching school; the first at Howard Seminary in West Bridgewater and the next two at the Pratt Institute High School in Brooklyn. Following this summer, they became engaged and were married in Crosswicks on September 19, 1901. Within a few years, three sons were born, Thomas Roger in Philadelphia on July 28, 1902, Hugh in Austin, Texas, on April 17, 1904 and Raymond Braislin in Philadelphia on May 5, 1910. In 1908, the young couple purchased the house on Buzzards Bay Avenue now owned by Norman T. Allen. No one could have foreseen then how closely the family was to become associated with the Woods Hole scientific community!

Professor Montgomery died of pneumonia on March 19, 1912, and his widow had to sell the Woods Hole property and go to work to support her family. At the University of Pennsylvania, Mrs. Montgomery worked as an assistant to the editor of the Journal of Biological Chemistry, Professor A. N. Richards. In 1915, she had a fellowship in Dr. McClung's laboratory to enable her to devote more time to work toward an advanced degree—work she was never able to complete. She next taught at Vassar for a year and then became an assistant professor at the University of Maine for the academic year 1918–1919. While there she taught vertebrate anatomy, histology and embryology, as well as undertaking some original studies of her own on the embryology of the chick.

Coming from a family of teachers, one might have supposed that Mrs. Montgomery would have continued in the family tradition, yet she had a drive and a lack of patience which made her temperamentally unsuited for such a career. Thus, in the fall of 1919, she became the "resident assistant librarian in immediate charge of the library" at the Laboratory, and in 1925, she was made Librarian.

It was due mainly to Mrs. Montgomery's respect for, and understanding of, research that the Library grew to its present stature. When she began in 1919 the entire Library had an annual budget of $2000 and was housed in Room 217 of the Crare Building. However, Mrs. Montgomery felt that she was assisting the work of the laboratory in a very real way and consequently her ambition for the Library knew no bounds. With persistence and the continuing help and interest of Dr. Frank R. Lillie and others, she accumulated an enviable collection, today consisting of some 75,000 volumes.

Although not a trained librarian, she knew the scientists' needs and developed an original, yet practical, system for filing journals, and at the same time expanded the reprint collection for circulation so that the journals themselves could always be available in the Library. On Mrs. Montgomery's retirement at the end of 1947, she had achieved her goal for building an outstanding library, which will long stand as a fitting tribute to her memory.

The building of her home on Whitman Road in 1923 marked the end of the years of her great personal struggle. By then her sons' education as a future business man, a doctor and a meteorologist-oceanographer were nearly completed. From then on she had time for sociability as well as work and she will be remembered for her genuine hospitality by many of the summer visitors at the Laboratory from this country and abroad.

1. The Staff, 1956

Philip B. Armstrong, Director, State University of New York, School of Medicine, Syracuse

Senior Staff of Investigation

A. P. Mathews, Professor of Biochemistry, *Emeritus*, University of Cincinnati

ZOOLOGY

I. Consultants

F. A. Brown, Jr., Professor of Zoology, Northwestern University
Libbie H. Hyman, American Museum of Natural History
A. C. Redfield, Woods Hole Oceanographic Institution

II. Instructors

Theodore H. Bullock, Associate Professor of Zoology, University of California, Los Angeles, in charge of course
John H. Lochhead, Professor of Zoology, University of Vermont
Norman A. Meinkoth, Associate Professor of Zoology, Swarthmore College
Grover Stephens, Assistant Professor of Zoology, University of Minnesota
John M. Anderson, Associate Professor of Zoology, Cornell University
Howard A. Schneiderman, Assistant Professor of Zoology, Cornell University
Martin W. Johnson, Professor of Marine Biology, Scripps Inst. of Oceanography, University of California, La Jolla
Morris Rockstein, Department of Physiology, New York University, Bellevue Medical Center

III. Laboratory Assistants

Charles H. Baxter, University of California, Los Angeles
Kent Christensen, Harvard University

EMBRYOLOGY

I. Instructors

Mac V. Edds, Jr., Associate Professor of Biology, Brown University, in charge of course
Paul B. Weisz, Associate Professor of Biology, Brown University
Nelson T. Spratt, Jr., Professor of Zoology, University of Minnesota
J. P. Trinkaus, Assistant Professor of Zoology, Yale University
Edgar Zwilling, Associate Professor of Genetics, University of Connecticut

II. Laboratory Assistant

Robert G. Beard, Indiana University

PHYSIOLOGY

I. Consultants

Merkel H. Jacobs, Professor of Physiology, University of Pennsylvania
Otto Loewi, Professor of Pharmacology, New York University, School of Medicine
Arthur K. Parpart, Professor of Biology, Princeton University
Albert Szent-Gyorgyi, Director, Institute for Muscle Research, Woods Hole
E. S. Guzman Barron, Associate Professor of Biochemistry, University of Chicago

II. Instructors

Stephen W. Kuffler, Associate Professor of Ophthalmology, Wilmer Institute, Johns Hopkins Medical School, in charge of course
Francis D. Carlson, Assistant Professor of Biophysics, Johns Hopkins University
Bernard D. Davis, Professor of Pharmacology, New York University, College of Medicine
Erik Zeuthen, Lecturer, Laboratory of Zoophysiology, University of Copenhagen
Raymond E. Zirkle, Professor of Radiobiology, University of Chicago
Herman M. Kalckar, National Institutes of Health
Max A. Lauffer, Professor and Head of Dept. of Biophysics, University of Pittsburgh
Andrew Szent-Gyorgyi, Independent Investigator, The Institute for Muscle Research

III. Laboratory Assistant

Paul Bernstein, Department of Zoology, Columbia University

BOTANY

I. Consultant

Wm. Randolph Taylor, Professor of Botany, University of Michigan

II. Instructors

Harold C. Bold, Professor of Biology, Vanderbilt University, in charge of course
Robert W. Krauss, University of Maryland
Paul C. Silva, Assistant Professor of Botany, University of Illinois

III. Laboratory Assistant

Raymond A. Galloway, University of Maryland

IV. Collector

Gina Arce, Vanderbilt University

MARINE ECOLOGY

I. Consultant

Alfred C. Redfield, Woods Hole Oceanographic Institution

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Charles Jenner, Associate Professor of Zoology, University of North Carolina

III. Assistant

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Lillie Fellow, 1956

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ECOLOGY

FERSAHL, SISTER JOHN BAPTIST, Fordham University
FOSTER, WALTER S., Colby College
GATES, JOHN O., Cornell University
GILBERT, ANN C., Columbia University
HIRCHINSON, VIVIENNE, Mount Holyoke College
JONES, SARAH R., Connecticut College
STRELECKI, RAYMOND F., Drew University
VAN DYK, N. JOANNE, University of New Hampshire
WEINSTOCK, AMMON, Brandeis University

3. FELLOWSHIPS AND SCHOLARSHIPS, 1956

Arsenious Boyer Fellowship:
FATHER WM. LYNCH

Lucretia Crocker Scholarship:
ROBERT ANDERSON, Botany Course
N. JOANNE VAN DYK, Ecology Course

The Gary N. Calkins Scholarship:
ROBERT ROBERTSON, Invertebrate Zoology Course

The Edwin Grant Conklin Scholarship:
HANS LAUFER, Embryology Course

Emma Coote Drew Scholarship:
ELOISE CLARK, Physiology Course

Bio Club Scholarship:
GRACE MOFFATT, Invertebrate Zoology Course

The Edwin Linton Scholarship:
ERNEST GUIGNON, Invertebrate Zoology Course

4. Tabular View of Attendance, 1952–1956

	1952	*1953*	*1954*	*1955*	*1956*
Investigators—Total	306	310	208	250	304
Independent	172	176	180	162	184
Under Instruction	38	37	20	9	20
Library Readers	49	46	52	54	50
Research Assistants	47	51	46	25	50
Students—Total	123	136	134	148	140
Zoology	55	55	56	56	55
Embryology	23	30	29	30	28
Physiology	27	31	28	30	30
Botany	11	11	12	19	18
Ecology	7	9	9	13	9
Total Attendance	429	446	432	398	444
Less persons represented as both students and investigators	2		5		2
	427	446	427	398	442
Institutions Represented—Total	149	155	136	129	130
By investigators	92	90	104	95	97
By students	57	65	32	34	33
Schools and Academies Represented					
By investigators	1		2	3	3
By students	3	1	1	2	1
Foreign Institutions Represented					
By investigators	7	15	11	8	9
By students	2	6	13	6	6

5. Cooperating and Subscribing Institutions, 1956

Cooperating Institutions

Amherst College
American Cancer Society
American Philosophical Society
Brooklyn College
Brown University
Bryn Mawr College
California Institute of Technology
Children's Hospital of Philadelphia
City College of New York
Colby College
College of Mt. St. Joseph on the Ohio
Columbia University
Columbia University, College of Physicians and Surgeons
Cornell University
Cornell University Medical School
Duke University
Elmira College
Emory University
Florida State University
Fordham University
Grass Foundation
Hahnemann Medical College
Harvard University
Harvard University Medical School
Indiana University
Institute for Cancer Research
Institute for Muscle Research
Johns Hopkins University
Johns Hopkins University Medical School
Lalor Foundation
Eli Lilly and Company
Marquette University
Morgan State College
Mount Holyoke College
National Institutes of Health
National Science Foundation
New York University—Heights
New York University, College of Medicine
New York University—Washington Square College
North Carolina State College
Northwestern University
Oberlin College
Office of Naval Research
Princeton University
Public Health Institute of New York
Rockefeller Foundation
Rockefeller Institute for Medical Research
Rutgers University

Saint Louis University
Sloan-Kettering Institute
Southwestern Medical College
State University of Iowa
State University of New York, College of Medicine, at Syracuse
Syracuse University
Temple University
Tufts College
University of Chicago
University of Connecticut
University of Illinois
University of Maryland School of Medicine
University of Michigan
University of Minnesota
University of Pennsylvania
University of Pennsylvania Medical School
University of Pittsburgh
University of Rochester
University of Virginia, School of Medicine
University of Wisconsin
Vassar College
Washington University
Washington and Jefferson College
Wellesley College
Wesleyan University
Western Reserve University
Yale University
Yale University Medical School

Subscribing Institutions

Acadia University
Armour and Company
Brandeis University
Chatham College
City College of New York
Drew University
Albert Einstein College of Medicine
Ethicon Corporation
Falmouth Medical Associates
Goucher College
Hamilton College
House of Good Samaritan
Hunter College
Massachusetts Institute of Technology
National Agricultural College
Purdue University
Michael Reese Hospital
Smith College
University of California
University of Florida
University of Maine
University of Massachusetts
University of New Hampshire
University of Oklahoma
University of Puerto Rico
University of Vermont
Veterans Administration Hospital
Washington University School of Medicine

6. Evening Lectures, 1956

June 29
Daniel Mazia ..... "Processes in cell reproduction"
July 6
Donald R. Griffin ..... "Listening in the dark"
July 13
Harry Grundfest ..... "The different kinds of electrical responses and their significance to the organism"
July 20
Vincent du Vigneaud ..... "The posterior pituitary hormones"
July 27
W. D. McElroy ..... "Recent developments in the biochemistry of light emission"
August 3
F. E. Lehmann ..... "Cytoplasmic organization and developmental physiology of the egg of Tubifex"
August 10
V. B. Wigglesworth ..... "The insect cuticle"
August 17
Alberto Stefanelli ..... "The life cycle of neurons"
August 24
Keith R. Porter ..... "The submicroscopic morphology of protoplasm"

7. Tuesday Evening Seminars, 1956

July 10

Joan Wolff and Roberts Rugh ....."The relation of gonad hormones to x-irradiation sensitivity in mice"

Paul R. Gross ....."Amphibian yolk: chemistry and ultrastructure"

Sylvan Nass ....."Amphibian yolk: the phosphoprotein phosphatase system"

8. Members of the Corporation, 1956

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V. REPORT OF THE LIBRARIAN

In 1956, the number of currently received journals totalled 1575 (48 new). Of these titles, there were 471 (6 new) Marine Biological Laboratory subscriptions; 607 (12 new) exchanges and 182 (15 new) gifts; 81 (9 new) were Woods Hole Oceanographic Institution subscriptions; 184 (4 new) were exchanges and 50 (2 new) were gifts.

The Laboratory purchased 60 books, received 77 complimentary copies (12 from authors and 65 from publishers), and accepted 25 miscellaneous gifts. The Institution purchased 23 titles and received 5 gifts. The total number of new books accessioned amounted to 190.

By purchase the Laboratory completed 14 journal sets and partially completed 13. The Institution completed four sets and partially completed three. Volumes and numbers received by gift and by exchange completed four sets and partially completed 16 sets.

There were 6112 reprints added to the collection, of which 2326 were of current issue.

At the end of the year, the Library contained 66,590 bound volumes and 202,201 reprints.

The Library sent out on inter-library loan 192 volumes and borrowed 74 for the convenience of the investigators.

Dr. E. Newton Harvey's collection of reprints was processed in 1956 and several thousand were added to the Library's collection. The duplicate material was presented to the University of North Carolina Library.

At the close of the year, Dr. Albert I. Lansing was influential in having an accumulation of reprints sent from the University of Pittsburgh. These will be processed in 1957.

Grateful acknowledgment is extended to Drs. Alfred C. Redfield, Henry Stommel, Ethel B. Harvey, Roberts Rugh, P. W. Whiting, Wm. R. Amberson, Dorothy Wrinch, Helen W. Kaan, and to the Tompkins-McCaw Library, Medical College of Virginia, for valuable and useful contributions of books, reprints and old photographs.

During the summer, the Library Committee, fully aware of the demand for an increase in the purchase of books, took action in securing a larger appropriation for this purpose. A Library Advisory Committee was appointed to recommend titles for purchase in 1957. There are 23 persons on this Committee and it is hoped a very substantial increase in book acquisitions will be realized. Extra funds are also expected in 1957 for the binding of back periodical volumes—work that has been neglected in order to keep the current binding up to date. The steady increase in the number of current periodicals now being published has also made it necessary to request an increase in the budget for 1957.

In August, Mrs. Priscilla B. Montgomery, the former Librarian, passed away. It is due to her foresight during the early days of the Library's rapid growth, that the present system, initiated by her, has proved to be an adaptable and an efficient one. The Library is a very fitting memorial to the loyal service and painstaking work displayed by her during the years 1919–1947. (A memorial paper is contained in the Laboratory's report).

Respectfully submitted,

DEBORAH L. HARLOW,
Librarian

VI. REPORT OF THE TREASURER

The combined market values of securities for the General Fund and the Library at December 31, 1956, amounted to $1,472,265.00 as compared with the total of $1,500,773 as of December 31, 1955. The average yield on the securities was 3.76% of market value and 5.55% of book value. The total uninvested principal cash in the above accounts as of December 31, 1956, was $1,551.72. The securities list held in the Endowment Funds appears in the auditor's report.

The pooled securities had a market value at the end of the year of $242,759.00 with uninvested principal cash in the amount of $430.12. The book value of the securities in this account was $223,341.33. The average yield on market value was 3.77% and 4.10% on book value.

The proportionate interest in the Pooled Fund account of the various Funds as of December 31, 1956, is as follows:

Pension Fund	16.680%
General Laboratory Investments	60.619
Other:	
Bio Club Scholarship Fund	1.768
Rev. Arsenious Boyer Scholarship Fund	2.161
Gary N. Calkins Fund	2.024
Allen R. Memhard Fund	.392
F. R. Lillie Memorial Fund	6.824
Lucretia Crocker Fund	7.391
E. G. Conklin Fund	1.252
M. H. Jacobs Scholarship Fund	.889

During the year we received a gift entitled "Frank R. Lillie Fellowship Fund" consisting of 800 shares of Crane Company common stock. While we can change the investments in this account, it must be kept intact. It was therefore not eligible to be included in the Pooled Fund account. Accordingly, a special custodian account was established for these securities. We are also holding in this account the securities of the General Biological Supply House.

The pledge of $8,000 par value government bonds still remains to secure the loan of the MBL Club which has been reduced over the years from $7,000 to $2,330 at the year end.

Donations from MBL Associates for 1956 were $5,255.00 as compared with $2,940.00 for 1955. Gifts from individuals for unrestricted use were $679.00. Foundations, societies and companies donated $25,500.00. The $6,677.83 balance of the $50,000 from the National Science Foundation for hurricane rehabilitation has been used to cover delayed repairs to the plant, attributed to the hurricane.

The apartment house rentals during the winter months amounted to $2,902.30. The cost of heating was $922.19 leaving a balance of $1,980.11 to be applied against the general expenses of lighting, water and insurance.

We were able to purchase short term treasury bills to activate some of the cash in the Falmouth account arising from the donations from foundations for anticipated construction, the payment for which was scheduled at a later date. The purchase of $50,000 U. S. Treasury bills was made in November, 1956 to run for 90 days. The interest earned on this purchase was $330.50.

Lybrand, Ross Bros. and Montgomery have examined our books and submitted financial statements for examination.

Following is a statement of the auditors.

To the Trustees of the Marine Biological Laboratory, Woods Hole, Massachusetts:

We have examined the balance sheets of the Marine Biological Laboratory as at December 31, 1956, and the related statements of operating expenditures and income and of current fund for the year then ended. Our examination was made in accordance with generally accepted auditing standards, and accordingly included such test of the accounting records and such other auditing procedures as we considered necessary in the circumstances.

In our opinion, the accompanying financial statements present fairly the assets, liabilities and funds of the Marine Biological Laboratory at December 31, 1956, and the expenditures and income for the year then ended.

LYBRAND, ROSS BROS. & MONTGOMERY

Boston, Massachusetts

MARINE BIOLOGICAL LABORATORY

Balance Sheet

December 31, 1956

Investments

Investments held by Trustee:	
Securities, at cost (approximate market quotation $1,472,265)	$ 995,086
Cash	1,552
	996,638
Investments of other endowment and unrestricted funds:	
Pooled Investments, at cost (approximate market quotation $242,759)	223,341
Other investments (Note A)	75,085
Cash	6,690
Accounts receivable	6,838
	311,954

Plant Assets

Land, buildings, library and equipment (Note B)	2,424,492
Less allowance for depreciation (Note B)	991,930
	1,432,562

Current Assets

Cash	100,045
U. S. Treasury bills, face value $50,000, due 2/15/57 temporarily invested pending expenditures for rehabilitation of Supply Department building and certain laboratories (approximate market quotation $49,800)	50,000
Common stocks, at market value at date of gift	5,728
Accounts receivable ($12,880 from U. S. Government)	25,535
Inventories of specimens and Bulletins	58,269
Prepaid insurance and other	15,980
	$2,996,711

Notes:

A—The Laboratory has guaranteed a note of approximately $2,400 of the M. B. L. Club and has pledged as security therefor bonds with an original cost of $7,900 included in other investments.

B—The Laboratory has since January 1, 1916 provided for reduction of book amounts of plant assets and funds invested in plant at annual rates ranging from 1% to 5% of the original cost of the assets.

MARINE BIOLOGICAL LABORATORY

Balance Sheets

December 31, 1956

Endowment Funds

Endowment funds given in trust for the benefit of the Marine Biological Laboratory		$ 996,638
Endowment funds for awards and scholarships:		
Principal	$ 62,861	
Unexpended income	1,420	64,281
Unrestricted funds functioning as endowment		206,378
Retirement fund		41,824
Pooled investments—accumulated gain or (loss)		(529)
		311,954

Plant Liability and Funds

Mortgage payable on demand, 5%		5,000
Funds expended for plant, less retirements	2,419,492	
Less allowance for depreciation charged thereto	991,930	1,427,562
		1,432,562

Current Liabilities and Funds

Accounts payable	13,653
Unexpended balances of gifts for designated purposes	11,540
Advance payments on research contracts	83,189
Current fund	147,175
	$2,996,711

MARINE BIOLOGICAL LABORATORY

Statement of Operating Expenditures and Income

Year Ended December 31, 1956

Operating Expenditures

Direct expenditures of departments:	
Research and accessory services	$161,259
Instruction	33,186
Library, including book purchases	28,631
Biological Bulletin	15,902
	238,978
Direct costs on research contracts	55,343
Administration and general	48,744
Plant operation and maintenance	76,926
Hurricane emergency repairs	6,678
Dormitories and dining services	131,464
Equipment purchased from current funds	2,850
	560,983
Less depreciation included in plant operation and dormitories and dining services above but charged to plant funds	36,424
	524,559

Income

Direct income of departments:	
Research fees	44,507
Accessory services (including sales of biological specimens $66,571)	94,978
Instruction fees	17,174
Library fees and income	6,625
Biological Bulletin, subscriptions and sales	18,148
	181,432
Reimbursement and allowance for direct and indirect costs on research contracts	56,429
Dormitories and dining services income	108,375
	346,236
Investment income	81,501
Gifts for current use	124,062
Sundry income	376
Total current income	552,175
Excess of income	$ 27,616

MARINE BIOLOGICAL LABORATORY

Statement of Current Fund

Year Ended December 31, 1956

Balance January 1, 1956	$119,559
Excess of income over operating expenditures, 1956	27,616
Balance December 31, 1956	$147,175

MARINE BIOLOGICAL LABORATORY

Summary of Investments

December 31, 1956

	Cost	% Total	Approximate Market Quotations	% of Total	Investment Income 1956
Securities held by Trustee:					
General endowment fund:					
U. S. Government bonds	$ 97,052	11.7	$ 93,462	7.7	$ 4,299
Other bonds	406,226	48.9	409,437	33.6	8,990
	503,278	60.6	502,899	41.3	13,289
Preferred stocks	85,788	10.3	71,500	5.8	3,370
Common stocks	241,652	29.1	644,556	52.9	29,350
	830,718	100.0	1,218,955	100.0	46,009
General Educational Board endowment fund:					
U. S. Government bonds	31,037	18.9	30,164	11.9	1,096
Other bonds	65,925	40.1	53,200	21.0	1,276
	96,962	59.0	83,364	32.9	2,372
Preferred stocks	27,281	16.6	24,274	9.6	1,130
Common stocks	40,125	24.4	145,672	57.5	5,807
	164,368	100.0	253,310	100.0	9,309
Total securities held by Trustee	$995,086		$1,472,265		$55,318

MARINE BIOLOGICAL LABORATORY

SUMMARY OF INVESTMENTS—*Continued*

December 31, 1956

	Cost	% Total	Approximate Market Quotations	% of Total	Investment Income 1956
Investments of other endowment and unrestricted funds:					
Pooled investments:					
U. S. Government bonds	$ 37,800	16.9	$ 34,119	14.1	$ 905
Other bonds	93,936	42.1	90,628	37.3	2,527
	131,736	59.0	124,747	51.4	3,432
Common stocks	91,605	41.0	118,012	48.6	5,439
	223,341	100.0	$ 242,759	100.0	8,871
Other investments:					
U. S. Government bonds	7,920				220
Common stocks	43,600				20,613
Real estate and mortgage	23,565				
	75,085				20,833
Total investments of other endowment and unrestricted funds	$298,426				$29,704
Total investment income received					85,353
Custodian's fees charged thereto					(412)
					$84,941

THE BREEDING OF POLYCHAETOUS ANNELIDS NEAR PARGUERA, PUERTO RICO

M. JEAN ALLEN [1]

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The writer set out to accomplish an embryological problem involving some of the polychaetous annelids of Puerto Rico. No information on the breeding habits of this group was available. To obtain suitable embryological material, therefore, it was necessary to determine the breeding periods of some of the species which were readily accessible. The information obtained is thus incidental to the main problem. Several people, however, have expressed an interest in its publication, since apparently little is known concerning the breeding habits of tropical marine forms. The information may be of some value to others interested in studying tropical or subtropical marine forms.

Observations and collections were made throughout the year. Night collections were made at the laboratory dock on the island of Magüeyes, Parguera, Puerto Rico, with a reflector bulb attached to an extension cord, and a dip net, or from a boat over nearby coral reefs with the aid of a reflector light attached to a battery. Some of the former collections were made throughout a full year; collections over the reefs were made from March to December, 1955. Collections made during the day were largely from the shallow seas over coral reefs, and along the edge of mangrove islands. The waters of this area of the Caribbean are relatively warm throughout the year, being somewhat cooler during the "winter" months. The temperature of the surface water goes as high as 87° F. at the laboratory dock (September 1, 1955). The tides in this area show little variation in height, the difference between the lowest low tide and the highest high tide rarely exceeding 41 cm. in any month.

Dr. Olga Hartman and Dr. Marian H. Pettibone were kind enough to identify the species here described. I am most grateful for their generous help, and also wish to express my appreciation to Mr. Donald Erdman who kindly assisted with many of the night collections, and to those individuals of the College of Agriculture who were so cooperative in putting the facilities of the Marine Institute and of the Department of Biology at my disposal.

The polychaetes on which observations have been made are arranged by families for convenient reference. Throughout the paper, one asterisk (*) refers to those species identified by Dr. Hartman, and two asterisks (**) to those identified by Dr. Pettibone.

FAMILY SYLLIDAE. *Autolytus ornatus* (Verrill) *. The female or sacconereis stage of the red-banded *Autolytus* may be collected at the dock at night the

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year round with the possible exception of the end of January and the beginning of February. It was found to be most abundant during the last half of May and June, during July (particularly during the middle of the month), and the first part of September. Individuals rise from a depth of several feet to the surface within the circle of light. When abundant, as many as 50 have been collected with one dip of the net, and as many as 165 have been collected within an hour. The female stage of this polychaete measures approximately 13 mm. However, individuals appear about half this size as they tend to curl ventrally around an egg sac in which developmental stages are borne. Before being released, white spherical eggs, approximately 85 micra in diameter, are packed within the coelom for the entire length of the body. Their development will be considered in a subsequent paper. The male (polybostrichus) stage of this syllid has been observed only twice. This was not at the dock where the sacconereis stage collects but over a reef on the nights of July 26, when 8 specimens were obtained, and September 21, 1955, when 2 specimens were collected.

In a series of papers written in 1951, Fauvel describes some polychaetous annelids from the Gulf of Tadjoura (French Somaliland) caught by M. J.-L. Dantan during night fishing with a light in the months of January, February, and March, 1933 and 1934. The Gulf of Tadjoura is closer to the equator than is Puerto Rico so presumably the water would be very warm the year round. Fauvel (1951a) notes that during night fishing in the gulf the syllids and nereids were the most abundant and most interesting of the species collected although other epitokal species were obtained too. The collecting in Puerto Rico also yielded primarily syllids and nereids. Fauvel (1951b) describes a number of species of syllids including *Autolytus*. It is interesting to note, however, that the polybostrichus stage of *Autolytus* was caught in enormous quantities (many hundreds) while the sacconereis stage was relatively rare (Fauvel, 1951a). As described above, the reverse was true in the Puerto Rican waters (although "enormous quantities" were not obtained), the male stage rarely being observed. From a plankton collection Thorson (1946) reported three sacconereis specimens of *A. prolifer* with larvae close to hatching stage in the ventral egg sac and one polybostrichus stage of the same species.

The temperature of the surface water from which *A. ornatus* was collected at Parguera, Puerto Rico, probably never goes below 75° F., even in January. It is interesting to note Bumpus (1898a) has reported that at Woods Hole, Massachusetts, during the month of March, 1898, *Autolytus cornutus* was frequently taken with eggs, the temperature ranging from 38° F. at the beginning of March to 43° F. at the end. Mead, in 1898, reported that *Autolytus* (species unspecified) with egg clusters attached was regularly taken in his tow at Woods Hole, usually 3 or 4 at a time, during the early part of April (water temperature approximately 41 to 42° F.). Bumpus (1898b) noted that the tow-net on May 7 brought in *Autolytus* but without eggs; on May 10th he noted several egg-bearing individuals, and on the 11th a male swimming about and finally fastening to a female with his jaw. Throughout the latter portion of May, *Autolytus* was abundant. In his report for June, July, and August, Bumpus (1898c) notes that *Autolytus* and other syllids which were frequently taken at Woods Hole had ventral egg sacs.

In the cold waters of Point Barrow, Alaska, both female stolons (with egg sacs) and male stolons have been reported for several species of *Autolytus* (Pettibone, 1954). This material was collected by Dr. G. E. MacGinitie of the Arctic

Research Laboratory. While the writer was still collecting *A. ornatus* in Puerto Rico, the following very interesting description of *Autolytus fallax* from the Point Barrow collection was published (MacGinitie, 1955, p. 138): "The female sacconereis stages bearing egg sacs were taken through the ice from January 25 to May 17, 1950. On March 29, 1950, there were hundreds of these worms, with egg sacs, swimming in the water at the 80-foot plankton hole and on April 7, 1950 (1.9 miles from shore) when the slush ice was removed from a 4-foot-wide lead, hundreds were welling up and swimming around. The worms were about 10 mm. long and the egg sacs 2.5 mm. long." It thus appears that *Autolytus* is able to tolerate a very wide temperature range for breeding, and that in the warm waters of Puerto Rico *A. ornatus* breeds all the year round with the possible exception of late January and early February. Hartman (1951) remarks that both sacconereis and polybostrichus stages of *Autolytus brevicirrata* Winternitz may be taken in plankton during the summer. Dales' comments (1951) on the breeding season of *A. prolifer* (*A. cornutus?*—see Pettibone, 1954) indicate that the breeding habits of this species may be similar to those of *A. ornatus*.

The sacconereis stage of another *syllid*, unidentified as yet, was collected occasionally along with *A. ornatus*. This species is yellow in color, is not banded, and has smaller eggs and larvae (various stages were collected) than those of *A. ornatus*. Thus far it would appear that this species cannot be collected in sufficient numbers for embryological purposes.

FAMILY NEREIDAE. *Nereis riisei* Grube*. Both male and female heteronereids of this species have been obtained in the evenings, both at the dock and over reefs, during most of the year. No real swarming, such as has been described for some species of *Nereis*, has been noted. Usually only one to a few individuals have been observed in an evening. The largest number observed was within an hour's time (between 8:00 and 9:00 P.M.) on March 23. Shedding does not ordinarily occur when the worms are isolated but only after a male and female are put together. The spherical eggs, approximately 190 micra in diameter, have numerous oil droplets as is characteristic of other nereid eggs. Results following attempts at artificial fertilization varied. In some cases no shedding occurred; in others, shedding of gametes occurred without development; in others, development ceased during cleavage; and in successful cases the eggs developed into larvae with three external sets of setae within three days. Results suggest that the height of the breeding period of this form extends from the last week in July to the beginning of September. None were obtained during four observations throughout June, with the exception of June 27 when one individual was observed. None were observed during the last part of September and October, 1955 (a few were collected on October 7, 1954). One was observed on November 7, none on November 14, and two (one with posterior end missing) on November 22, 1955. Occasional individuals were collected throughout the other months of the year.

A number of nereids have been described by Fauval (1951a-d) from the collection from the Gulf of Tadjoura but this group did not include *Nereis riisei* Grube. The heteronereid stages of *N. riisei*, collected at Parguera, Puerto Rico, are in size, color, and behavior under the light, reminiscent of *Nereis succinea* (Leuckart), commonly called *Nereis limbata* (Ehlers), found at Woods Hole, Massachusetts. *N. riisei* differs, however, in that the males observed were larger than the females and no real swarming occurs. This is in even greater contrast to the spectacular

behavior of the *Nereis succinea* occurring in eastern Canadian waters (Berkeley and Berkeley, 1953). Swarming in the case of *N. succinea* (at least, in the Woods Hole area) seems to be more closely associated with the lunar cycle (Lillie and Just, 1913) than is the appearance of the heteronereid stage of *N. riisei* in Puerto Rican waters.

Ceratonereis mirabilis Kinberg **. Small heteronereids of this species (approximately 7 mm. in length) were collected at night over coral reefs. They were never observed at the dock. Night collections over reefs were begun on March 11, 1955, when the only suggestion of real swarming of this species was observed. The heteronereids shed their gametes when isolated. Males appear yellow in color prior to shedding and pinkish (posterior portion) after shedding. Females appear whitish before and pinkish after shedding. The eggs, approximately 120–125 micra in the long diameter, are flattened spheres suggesting biconvex discs. Artificial fertilization was attempted whenever both males and females were collected. It was always successful. Embryos swim within 8 hours after fertilization, trochophores develop within 12 hours after fertilization, and beautiful larvae with three bushy sets of setae develop within 2½ days. A few have been kept alive for 22 days but developed no additional setae. Collections were made from March 11 to December 14, 1955, but both sexes were observed only during the new moon each month with the exception of March 11, three days after full moon, when the best batch of eggs was obtained (to date this exception has not been explained), and three days before the first quarter in September when a few were obtained. It should be noted that only one sex in the heteronereid form was collected during the new moons of June, September, and December.

Fauvel (1951c) describes several species of *Ceratonereis*, including *C. mirabilis*, from the night collections in the Gulf of Tadjoura. In several species, only heteronereid males were caught. In the case of *C. mirabilis*, the male epitoke was caught in numbers with a few females (mostly in fragments). Fauvel points out that while this species is very widely distributed in all warm regions of the Atlantic, of the Pacific and particularly of the Indian Ocean (with which the Gulf of Tadjoura is indirectly connected), the epitokal form has been only rarely encountered, the few females being mainly fragments. In the Puerto Rican waters the writer was apparently fortunate in obtaining both sexes in the heteronereid form for embryological purposes.

*Nereis sp.***. This is a small pink species, measuring approximately 2 cm. in length, with a rather thick body. This worm was frequently collected along with *Ceratonereis* and other small nereids, but insofar as can be determined only the male heteronereids were observed. This has been the experience of other investigators (see Fauvel, 1951a-d).

Platynereis dumerilii (Audouin and M. Edwards) **. This small pink heteronereid, flattened dorso-ventrally, is usually somewhat smaller than the preceding. The heteronereid stage was collected frequently along with *Ceratonereis* and *Nereis sp.* Again, only male heteronereids were detected. Fauvel (1951d) describes several species of *Platynereis* but not *P. dumerilii*. It is interesting to note that in the *Platynereis dumerilii* at Naples which breeds from October through May spectacular swarming has been observed after the full moon in May (Just, 1929), while only a few heteronereids were observed in Puerto Rico and these during the last quarter of the moon and the new moon (several observations). The het-

eronereid stages of *P. dumerilii* were also observed between 8:00 and 11:00 P.M. in March, 1955, during the dark of the moon (no moon) on Loch Hourn, Scotland (Dr. L. R. Fisher, personal communication, 1957). These observations suggest that *P. dumerilii* is able to tolerate a wide temperature range for breeding.

Nereis allenae (Pettibone). Only two heteronereids of this small species (approximately 1 cm. long) have been collected, one on August 25 (first quarter of the moon) and one on September 15 (new moon), 1955. Both were collected during the evening from the shallow water over the reef between the Marine Laboratory and Caballo Blanco Island, Parguera, Puerto Rico. This was the first time that the writer had observed polychaete eggs laid in short strings resembling blue-green algae. The eggs made up a single row of cells (approximately 30 eggs in some strings). Within about an hour after laying, the eggs became isolated due to the dissolution of the substance (jelly?) holding the eggs together. The eggs after fixation measure approximately 160 micra in diameter. This polychaete is a new species (Pettibone, 1956).

Several other species of nereids are represented by heteronereids (including egg-laying females) in night collections but they have not, as yet, been identified.

FAMILY GLYCERIDAE. *Glycera ?sphyrabrancha* Schmarda **. On the evening of October 31, 1955, between 8 and 8:40 P.M.,two females, 28 cm. and 23 cm. long, respectively (when fixed), and a long male segment of this species were observed swimming in surface water at the dock light. They were caught in a dip net and isolated in finger bowls, whereupon the shorter female and the male segment shed their gametes. The eggs (resembling flattened tops and approximately 125 micra in diameter) are whitish, with a large central germinal vesicle (50 micra in diameter) and finely granular cytoplasm. They are liberated in two streams, apparently from two pores about midway down the body. Two drops of diluted very active spermatozoa were used to inseminate the eggs. At approximately 9:30 P.M. another female was observed and caught with the dip net. The relatively few eggs suggested she may have shed already. Within 1 hour and 40 minutes after insemination a number of the eggs of the first female were at the 2- to 4-cell stage (mostly the latter) but a number had not cleaved. Within 11 hours many ciliated swimmers were observed in the dish. Within the next 24 hours larvae with a prominent prototroch had formed and within the next 24 hours (age, 2½ days) elongating larvae with a differentiating gut had developed.

EUNICIDAE. *Eunice ?pennata* (Müller) **. These slender worms, approximately 7½ cm. long, build small sand tubes, externally covered with shell. They are found on the under surface of coral in shallow water. When the coelom of a female is packed with eggs, the posterior half of the body appears green due to the pigment within the eggs. The eggs, approximately 175 × 160 micra in diameter, are oval with a prominent germinal vesicle. The posterior half of males with spermatozoa appears cream in color. Not many specimens have been collected, but females with eggs have been observed in February, March, April, May (one female was observed over a reef on the evening of May 6), September, and November, 1955. The few attempts at artificial fertilization were unsuccessful.

Eunice (*Nicidion*) *sp.***. This is a small species about the size of *E. ?pennata*. The worms are found on the under surface of coral in association with *E. ?pennata* tubes, or close to them, and may occur in clumps of several individuals twined about one another. The eggs (observed only in September) are bright pink and may be

observed readily through the body wall. This species has been observed only in September, October, and November, but no definite attempts were made to look for it. Fauvel (1951d) identified several species from the Gulf of Tadjoura collection, including *Eunice* (*Nicidion*) *edentulum* which he describes as a cosmopolitan and widely distributed species living in the Indian Ocean, Pacific, and Atlantic.

*Lysidice sp.***. Between 7:30 and 9:00 P.M. on the evening of December 14, 1955, a number of worms were observed in the circle of light swimming in the shallow water over a reef near the laboratory and several were shedding gametes. The adults were isolated and the eggs were inseminated artificially. In about three hours spherical surface swimmers were observed and 16 hours after insemination orange-red eyespots were visible. This species also was collected in November but shedding was not observed. Fauvel (1951d) identified *Lysidice collaris* from the Gulf of Tadjoura and described it as being widely distributed in warm seas.

AMPHINOMIDAE. *Hermodice carunculata* (Pallas) *. This large fireworm is rather plentiful in the Puerto Rican area and is locally referred to as a "marine centipede." It has been observed during the daytime lying about on reefs in shallow water or on the sandy to muddy bottoms surrounding mangrove islands. An occasional individual was checked for gametes at least once a month from January through June, 1955, by making a small slit in the body wall. No gametes were observed in this period except on January 13 when an injured female shed many orange-colored eggs, approximately 100 micra in diameter. In November, 1955, a few specimens were again checked for gametes. Eggs were observed (though not very abundant) in one female on November 8, and in another on November 29.

Eurythoë complanata (Pallas) *. This is another common "marine centipede" often collected along with *H. carunculata* on shallow reefs. Eggs have not been observed but very small worms have been collected early in November (one measured 19 mm. and a second 13 mm. when fixed). Fauvel (1951a) identified one specimen of this species taken from the Reef of Ambouli, noting that it is found in all tropical seas. Both this species and the preceding are found in the Gulf of Mexico along the coast of Florida (Hartman, 1951).

Notopygos crinita Grube **. This species of fireworm has been observed only in May. On the evening of May 13, 1955, two males and one female were observed swimming over a reef. When isolated in finger bowls, they shed immediately. The eggs fertilized readily, developing into much flattened, top-like trochophores within 14 hours. Four days after insemination, they had developed into weird-looking larvae with two sets of much elongated glass-like setae. The larvae died within the next two days. On the evening of May 20 a small male was collected similarly. It did not shed until taken to the laboratory. No other individuals were observed.

FAMILY POLYNOIDAE. *Chaetacanthus magnificus* (Grube) *. Often one to several of these worms were collected incidentally on coral reefs in shallow water from September, 1954, to May, 1955, but no shedding was observed nor were gametes seen when the body wall was pricked. Later (November 14, 1955) a few more were collected. Upon puncturing the body wall one of these shed a few whitish, opaque, almost spherical eggs, approximately 100 × 85 mm. in diameter.

FAMILY TEREBELLIDAE. *Thelepus setosus* (Quatrefages) *. This species is found in tubes, covered by sand and shells, on the under surface of coral in shallow water. Pink, disc-shaped eggs (the larger measuring approximately 185

micra in diameter) are readily observed through the body wall moving about within the coelomic fluid. A number of individuals have been collected each month from November, 1954, to November, 1955, at various phases of the moon (collections were made during the day) but no fertilization has been attained. Individuals when collected were isolated and checked periodically for shedding. Changing the temperature, and also the sea water in which they have been kept, was done consistently but did not prove successful in stimulating shedding. In one instance, on March 31, a male shed active spermatozoa within 30 minutes after isolation; an attempt at artificial fertilization was unsuccessful. On September 10, one individual out of six shed many eggs on the day after it was collected (following two changes of temperature and of sea water). The breeding habits of *Thelepus* may be similar to those of its relative, *Amphitrite ornata* Verrill. Mead (1897) collected approximately 800 specimens of *A. ornata* from June through August and only rarely obtained ripe gametes. He stated (p. 229) that it "is useless to cut the animals open, for, if the sexual products are mature, they will be discharged, usually at about 6 o'clock in the evening, more often on the day of capture, sometimes the next day. The rarity of ripe specimens is partly compensated for by the enormous number of eggs which may be obtained from one female." Scott (1909) concluded that the height of spawning for *A. ornata* is in July and is closely related to the spring tide. As suggested by the above observations, no definite conclusions can be drawn, as yet, regarding the specific breeding habits of *Thelepus setosus*.

Eupolymnia crassicornis (Schmarda) *. This worm lives in a collapsible tube of mucus reinforced by pebbles, sand, shell, calcareous algae, etc. The breeding habits of this species probably are similar to those of *Thelepus*. Eggs, if plentiful enough, appear as lavender areas seen through the body wall. The color is due to the pigment in the ova. The larger of the disc-shaped eggs are approximately 175 micra in diameter. This species likewise has been checked the year round, from January to December, 1955, and treated in the same manner as *Thelepus*. On March 15, at 5:00 P.M., individuals collected that day were isolated, and by 8:00 P.M. two females had shed many eggs, and one a few eggs. By then, the germinal vesicle had broken and one male had shed active spermatozoa. Two batches of eggs were inseminated; one showed no development and the second had an occasional swimmer moving slowly within 3½ hours after insemination. All of these swimmers died within the next day and a half. Possibly the eggs were over-ripe. On June 27, another attempt at artificial insemination was unsuccessful. On September 11, six males shed active spermatozoa. Artificial insemination was tried although the female used had not shed normally. No development occurred. On November 15, one out of the six worms collected that morning had shed active spermatozoa by 6:00 P.M., and on November 29 one of two males shed at approximately 6:00 P.M.

SABELLIDAE. *Sabellastarte magnifica* (Shaw) **. This large feather duster lives in a membranous tube attached to coral in shallow water. Relatively few specimens have been checked the year round for gametes. The rather small eggs (approximately 55 micra in diameter) are whitish discs with a central germinal vesicle. They tend to shrink when freed from the coelom, suggesting that they are not osmotically balanced with the sea water. Eggs have been observed in August, September, and October, and males with active spermatozoa have been collected

in September, October, and November (one male, November 23). The few attempts at artificial fertilization were unsuccessful.

Sabella melanostigma Schmarda*. This species of feather duster, smaller and more slender than the preceding, lives in a slender membranous tube (covered with fine silt and mud) found attached to stones or shells or to mangrove roots. Individuals with eggs (most of the eggs shrivel in sea water) and motile spermatozoa were collected from January through May. No gametes were observed in the few specimens checked in June, July, and August. One check was made in September — two females examined had minute eggs; a male individual, considerably smaller than the females, had active spermatozoa. Both eggs and active spermatozoa were noted in October and November. The few attempts at artificial fertilization, scattered throughout the year, proved unsuccessful.

SERPULIDAE. *Spirobranchus tricornis* (Mörch) *. This small feather duster builds a calcareous tube in the common coral, *Porites porites*. The eggs, approximately 80–85 micra in diameter, are flattened spheres. They are relatively clear except for the orange pigment granules in the cytoplasm. The sex of individuals can often be determined with the naked eye if the coelom is packed with gametes. Females have bright orange tips (basal end), as the pigment in the mass of eggs shows through the body wall; males have whitish or creamish tips when packed with spermatozoa. This species has been checked for gametes in every month from September, 1954, to November, 1955. Only a few swimmers developed following attempts at artificial fertilization from November through February. A number cleaved following artificial fertilization from March through October, 1955. These developed into typical trochophores, some batches swarming with the ciliated swimmers. A member of this genus, *Spirobranchus giganteus* (Pallas), has been described by Fauvel (1951d) in the collection from the Gulf of Tadjoura. He notes that this species occurs in all intertropical regions of the Indian Ocean, Pacific, and Atlantic, mainly on coral reefs.

It is hoped that the positive results recorded here will prove useful to other investigators interested in tropical or subtropical marine forms. Negative results should be viewed with caution.

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ENCYSTMENT STAGES OF DICTYOSTELIUM [1]

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The genus *Dictyostelium* Brefeld (1869) is representative of the Acrasieae, a group of simple, cellular slime molds, wherein the life cycle consists of an amoeboid vegetative phase and a plant-like fruiting phase. The vegetative phase of these primitive micro-organisms is characterized by the independent movement and multiplication of free-living myxamoebae which feed by the ingestion and digestion of bacterial cells. The fruiting phase normally begins with the exhaustion of this food supply and is first evidenced by the coordinated inflowing of the myxamoebae to form wheel-shaped aggregates, or *pseudoplasmodia.* It attains its definitive expression as a portion of the myxamoebae thus assembled becomes transformed into vacuolate, parenchyma-like cells to form the upright stalk, or *sorophore,* whilst the remainder differentiate into capsule-shaped reproductive cells, or *spores,* to form the elevated spore mass, or *sorus,* of the slime mold fructification, or *sorocarp.* In most cultures of *Dictyostelium* grown under favorable cultural conditions, virtually all of the former vegetative myxamoebae enter directly into radiate pseudoplasmodia and subsequently differentiate into either stalk cells or spores (Olive, 1902; Raper, 1935, 1940a, 1940b; Bonner, 1944). However, marked exceptions to this behavioral pattern occur in certain species and strains.

Under some conditions, not wholly understood, many of the vegetative myxamoebae never enter the fruiting state (*i.e.,* aggregate to form pseudoplasmodia) but as individual cells enter an encystment stage. Such resting cells are termed *microcysts* (Fig. 1). This designation was first applied by Cienkowski (*fide* Olive, 1902) to describe those myxamoebae of *Guttulina* which under unfavorable conditions tended to form rounded protoplasmic bodies with definite ectoplasmic membranes. Olive widened the application to include other Acrasieae and stated that microcyst formation occurred under unfavorable conditions such as slow drying in hanging-drop preparations. In our investigations such individually encysted cells have been observed from time to time and in large numbers in agar plate cultures of *Dictyostelium mucoroides* Brefeld (1869), and with even greater frequency in *D. minutum* Raper (1941), *D. polycephalum* Raper (1956a, 1956b), *Polysphondylium pallidum* Olive (1901, 1902), and *Acytostelium leptosomum* Raper (1956b). They are believed to occur in greater or lesser numbers in all aging cultures.

An additional, multicellular encystment stage occurs in occasional strains of *Dictyostelium mucoroides* and in many isolates of *D. minutum.* These relatively complex structures arise by a morphogenetic process possibly alternative to normal

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sorocarp formation. Because of their larger dimensions and their multicellular origin and constitution, these bodies are termed *macrocysts* (Fig. 2). This designation was first applied by Raper in 1951 with reference to structures seen in certain cultures of *D. minutum* isolated from soil, but no description of the macrocysts was given. More recently, Cormier and Raper (1955) and Raper (1956b) have reported briefly concerning their formation and structure. It is of special interest that Brefeld, in his original paper on *D. mucoroides* (1869), described and illustrated as "dwarfed sporangia" structures which are believed to have represented macrocysts (Fig. 3).

The primary purpose of this investigation has been to extend our observations on these long neglected structures and to seek answers to three basic questions pertaining to them, namely: How are the macrocysts and microcysts formed? What conditions favor their development? What are the roles of these encystment stages in the life cycle of the Acrasieae? A resumé of our present knowledge of these matters is presented herewith.

Materials and Methods

Micro-organisms investigated

Several cultures of *Dictyostelium* were examined relative to the production and possible function of the macrocysts, including *Dictyostelium mucoroides* (Strains S–28b, NC–12, and WS–47) and *D. minutum* (Pur–8a and WS–56–2). *Dictyostelium mucoroides* was the slime mold studied most intensively, particularly strain S–28b which produces abundant macrocysts. Macrocysts were first observed and photographed in strain NC–12 when this slime mold was isolated in 1937 (Fig. 2).

Microcysts of several species of the Acrasieae were studied, including those of *Dictyostelium minutum* (WS–116b), *D. polycephalum* (S–4), *Polysphondylium pallidum* (WS–116c), and *Acytostelium leptosomum* (FG–12a). Those of *D. polycephalum* are illustrated in Figure 1.

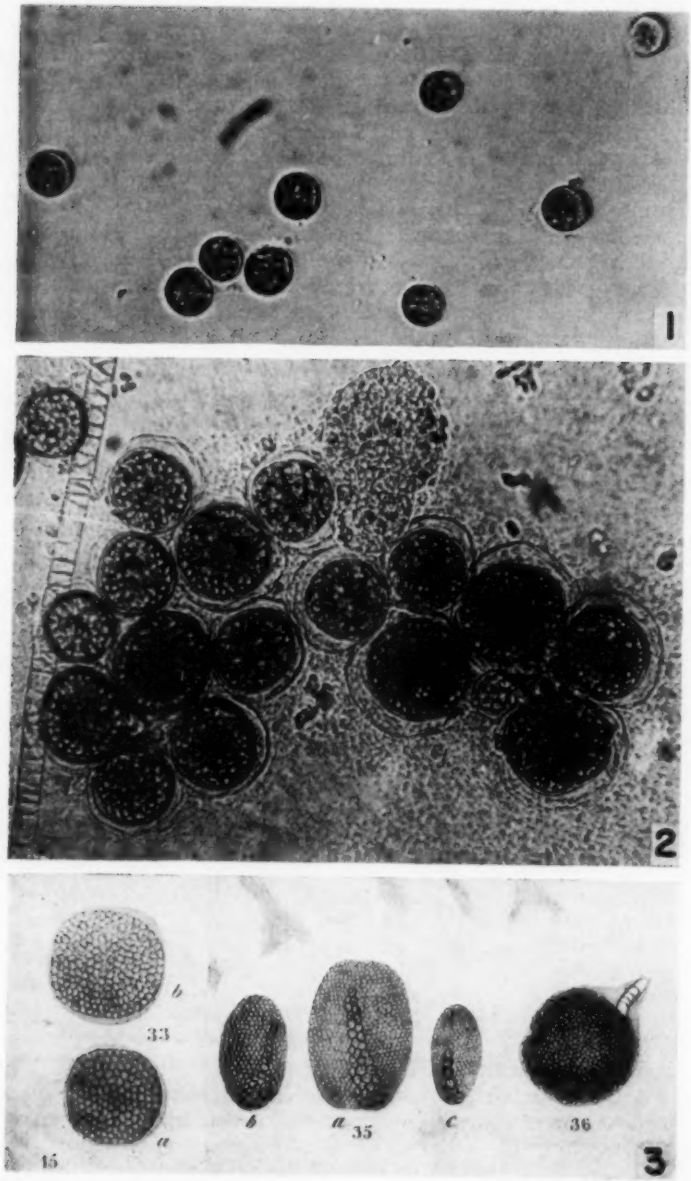
Different bacterial associates were investigated as food sources for the slime molds and for their possible influence upon macrocyst formation. Included among gram-negative species were *Escherichia coli* (No. B–281), *Aerobacter* sp. (Singh's strain), *Aerobacter aerogenes* (Sussman's strain), *Flavobacterium* sp. (DIF), *Serratia marcescens* (No. B–175), and *Pseudomonas fluorescens* (No. B–112); included among gram-positive species were *Bacillus megaterium* (No. B–160), *B. subtilis* (Sarles' strain), and *Sarcina lutea* (No. B–1018). *Escherichia coli*, the organism used most commonly, is a short rod which is mildly proteolytic and ferments both dextrose and lactose; the myxamoebae appeared to grow best when feeding upon this bacterium.

Cultivation of the slime molds

Environmental and cultural conditions, including (1) temperature, (2) culture media, (3) pH of substrate, (4) ammonium ion concentration, and (5) per cent relative humidity, were varied to determine their effect upon macrocyst formation in *Dictyostelium*.

Incubation temperatures used in most of the experiments were 10, 15, 20, 25,

PLATE I



and 30° C. The optimum growth temperature for the macrocyst-forming strains of *Dictyostelium mucoroides* was about 20° C., whereas that of the *D. minutum* isolates more nearly approximated 25° C.

Many different media were employed for the cultivation of the slime molds and the associated nutritive bacteria, which, for the most part, were patterned after substrates previously reported by Raper (1951). An agar medium containing 0.1% lactose and 0.1% peptone (0.1 L–P agar) was used most extensively since it provided the most reproducible growth and the most consistent macrocyst formation in *D. mucoroides* and in *D. minutum*. Horse dung-infusion and 0.05% uric acid agars [3] were also employed because of Brefeld's report (1869) that dung extract and uric acid induced spore germination.

The pH of the substrates employed for the conjoint growth of the slime molds and associated bacteria was varied by buffering with KCl, potassium acid phthalate, KH_2PO_4, and K_3BO_3 as recommended by Clark and Lubs (Clark, 1928). Bacteria were also pre-grown on buffered 0.1 L–P medium of varied pH and transferred to unbuffered medium prior to inoculation with the slime mold (Raper, 1951). Experiments to determine the possible effect of ammonium ions on growth and macrocyst formation in *Dictyostelium* were patterned after those reported by Cohen (1953).

Different relative humidities were obtained by placing specified concentrations of H_2SO_4 in water in desiccators, as reported by Wilson (1921). Two types of slide cultures were employed: (1) Maximov tissue culture slides containing one ml. 0.1 L–P agar inoculated with a mixed suspension of spores, or myxamoebae and macrocysts, of *D. mucoroides* and *E. coli* cells; (2) plain flat slides spread with 1.0 ml. molten 0.1 L–P agar and inoculated by on-flowing a mixed suspension of slime mold and bacteria. The latter slides were supported in an upright position by small wooden blocks during incubation.

Agar plate cultures were grown routinely in glass Petri dishes, to maintain a high per cent relative humidity, and incubated in the dark at varying temperatures. The plates were cross-streaked or completely smeared with the bacterial associate and then inoculated at the center with spores, macrocysts, or myxamoebae of the selected slime mold, the type of inoculum and bacterial associate being varied with the experiment.

[3] Dung decoction was made by autoclaving 100 grams of fresh horse dung/liter of water for twenty minutes at 15 pounds' pressure. The resulting decoction was filtered, solidified with 1.5% agar, and re-sterilized (final pH 6.1–6.35). Uric acid agar consisted of a 0.05% aqueous solution of uric acid to which 2.0% agar was added.

PLATE I

FIGURE 1. Microcysts of *Dictyostelium polycephalum*, representing individually encysted vegetative myxamoebae. × 530.

FIGURE 2. Macrocysts of *Dictyostelium mucoroides*, Strain MC–12, showing the characteristic habit and structure of these bodies; the cellular stalk of a normal sorocarp appears at the far left. × 250.

FIGURE 3. Illustrations from Brefeld's description of *Dictyostelium mucoroides* (1869), from left to right: "Dwarfed sporangia," which are believed to be identical with the macrocysts reported in this paper; "small sporangia" with "rudimentary stalks" in the spore-forming plasm; "sporangium" with a small stalk surrounded by an enclosing membrane. × 300.

Reagents

Several reagents were employed to establish the cellulosic nature of the walls of the macrocysts, namely:

(1) Chloroiodide of zinc solution was prepared by dissolving 30 grams of ZnCl, 5 grams of KI, and 0.89 gram of iodine in 14 ml. of distilled water (Stevens, 1916). The material to be tested was mounted in water, after which the reagent was applied to one edge of the cover glass and drawn under it by placing a piece of filter paper against the opposite edge. Cellulosic material stains violet-blue.

(2) Congo red was prepared as a 0.5% aqueous solution of the dye made alkaline by adding two or three drops of concentrated NaOH. In an alkaline solution this reagent stains cellulose red, which when put in HCl turns blue (Raper and Fennell, 1952). This is a presumptive but non-specific test for cellulose.

(3) Schweitzer's reagent was prepared by bubbling air through 60 ml. of NH_4OH containing 10 grams of fine copper turnings for one hour (Hodgman, 1951). Cellulose is quickly dissolved by this strong cuprammonium solution. In practice, the macrocysts were generally mounted in water and the reagent was applied by drawing it under the cover glass, hence diluting and advantageously slowing its action. A 72% aqueous solution of H_2SO_4 was also used as a cellulose solvent.

(4) A strong birefringence in polarized light was likewise interpreted as confirming the predominantly cellulosic composition of the macrocyst wall.

Nile blue sulfate was employed as a vital dye for staining myxamoebae and progressive developmental stages as reported by Bonner (1952). It was also incorporated into 0.1 L–P agar prior to inoculation with *E. coli* and the slime mold, *ca.* 3 ml./liter of a 0.5% aqueous solution being employed for this purpose. Used directly, or when added to the growth medium, it stained vegetative myxamoebae a very light blue, whereas aggregating myxamoebae and the cells of developing macrocysts assumed somewhat darker shades and appeared less granular.

Experimental Results

Origin and morphology of macrocysts

Macrocysts are flattened, irregularly circular to ellipsoidal multicellular structures, ranging from 25 to 50 μ in diameter. The myxamoebae which contribute to their formation appear normal for *Dictyostelium mucoroides* and *D. minutum* in every respect as they move and feed upon bacterial cells, re-dividing until the available food supply is exhausted. As this occurs, the myxamoebae begin to aggregate into pseudoplasmodia which, except for their generally limited dimensions, appear basically similar to other pseudoplasmodia that proceed to sorocarp formation (Fig. 4). However, instead of producing upright sorocarps, the myxamoebae comprising these aggregates remain in compact heaps and subsequently become surrounded by comparatively thick, cellulose walls. When only a few myxamoebae aggregate to form pseudoplasmodia, small macrocysts develop singly; when larger numbers of cells are involved, the macrocysts are somewhat larger and may occur in groups of varying size, ranging from small packets to

sheet-like ribbons, depending upon the number of myxamoebae massed together. In these larger aggregations, incipient macrocysts are delimited by the secretion of delicate cellulosic membranes around limited groups of cells more or less regularly spaced throughout the primary aggregate. This is followed by the subsequent deposition of thick, predominantly cellulosic walls, mostly circular to oval in pattern, that become the relatively rigid boundaries of the individual macrocysts. Commonly the secondary wall is laid down in general conformity with the primary membrane, but not infrequently two, three or even more macrocysts develop within an initial area of demarcation (Figs. 5–7).

Somewhat prior to the first evidence of secondary wall formation around the nascent macrocyst, cells in the center of the previously undifferentiated mass exhibit signs of modification and become surrounded individually by strongly refractive membranes. This process of cellular differentiation advances outward until all of the cells comprising the macrocyst are transformed into closely packed, seemingly firm-walled cells, termed *endocytes* (Figs. 8–9). The constitutive myxamoebae show no obvious orientation during the early stages of this process; but as differentiation proceeds, the peripheral and still amoeboid cells become conspicuously elongate as if appressed against the surface of the steadily enlarging body of endocytes (Fig. 6). Parallel with this progressive differentiation of the endocytes, but in a manner not yet understood, the whole body of functionally integrated cells succeeds in building around itself a tough and relatively heavy wall that is rich in cellulose. Significantly, the position of this wall is not determined by that of the thin primary membrane which initially delimited the bloc of myxamoebae that *might* collaborate in macrocyst formation; rather it is determined by the group(s) of cells which first differentiate as endocytes. This is clearly evident from the examination of primary aggregates of different dimensions. If the aggregate is small, the subsequently formed macrocyst wall will conform generally to that of the primary membrane. If the aggregate is relatively large, several centers of endocyte formation will arise simultaneously, and outward from these loci, cells will differentiate progressively to form separate and independent macrocysts, each with its own characteristic heavy wall but all contained within the primary membrane (Figs. 7–9, 25–27). The entire process of macrocyst formation is normally completed within 18 to 24 hours. Successive stages in macrocyst formation are illustrated in Figures 4 through 9.

The endocytes may be isodiametric or slightly elongate, ranging in size from about 3.6 to 4.8 μ in diameter. As observed within the macrocyst, and when first released by breaking the macrocyst wall, these cells normally appear polyhedral in outline, but soon become spheroidal or ellipsoidal when no longer compressed by adjacent endocytes (Fig. 9). For reasons still unknown they are appreciably smaller than the myxamoebae which enter the primary aggregate, the latter usually ranging between 6.0–8.0 μ in the unexpanded state. The smaller dimensions of the endocytes may result from a substantial water loss during their differentiation, or the contributing myxamoebae may possibly undergo division prior to the development of the refractive membranes which so strikingly distinguish them from other cells still amoeboid (Fig. 8).

Endocytes normally remain as distinct cellular entities within the macrocyst for a period of two or three weeks (Fig. 10), after which they commonly lose their identity and the protoplasmic content of the entire structure assumes a homo-

PLATE II. Origin and structure of macrocysts in *Dictyostelium mucoroides*, Strain S-28b.

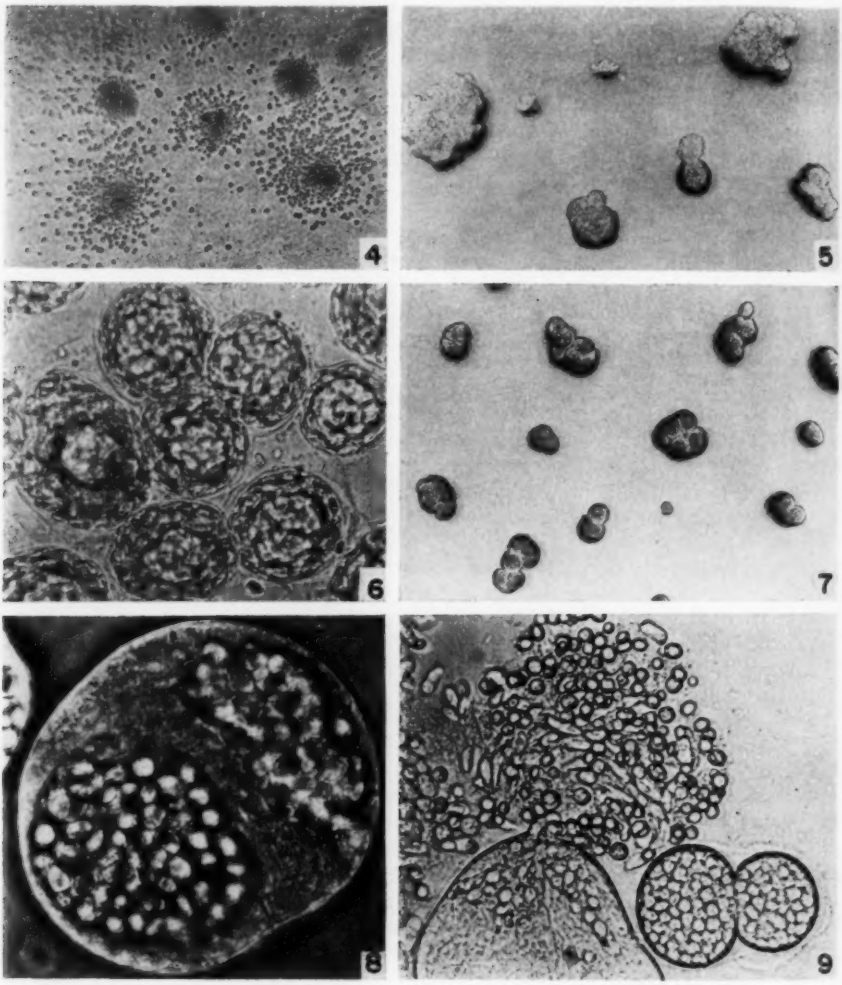


FIGURE 4. Small radiate pseudoplasmodia which lead to the formation of macrocysts. × 80.

FIGURE 5. Completed aggregations, consisting of irregular mounds of myxamoebae, prior to the formation of the primary membranes that delimit incipient macrocysts. × 80.

FIGURE 6. Enlarged view of a later stage in macrocyst development, showing incipient macrocysts and the orientation of their constituent myxamoebae. × 360.

FIGURE 7. More advanced stage showing clusters of differentiated endocytes centrally located in developing macrocysts. × 80.

FIGURE 8. Much enlarged view of two macrocysts in process of formation within a single

geneous appearance. With further aging, from four to six weeks, the apparently acellular content often shrinks to approximately 60–75% of its original volume, and in the form of a compacted, brownish mass occupies a central position within the partially empty macrocyst wall (Fig. 13). The surface of this central body appears slightly irregular and affords no clue to the presence of a continuous bounding membrane; rather it suggests a plasmolyzed and shrunken mass that has been subjected to uneven pressures and tensions during the process of contraction.

Many questions remain unanswered concerning the sequence of events leading to the advanced acellular structures just described. We have inadequate information concerning their true nature, and we have only incomplete knowledge of their significance in the life cycle of those slime molds where they occur. Nevertheless, a detailed study of macrocysts of different ages, and under many conditions, has revealed a considerable body of information concerning their development and behavior. If an endocyte-filled macrocyst is subjected to pressure in an aqueous mount, the heavy cellulose wall breaks, much as a hollow rubber ball, and the endocytes pour out, undergoing the limited changes in shape already noted but retaining their identity as relatively firm-walled cells. In contrast, if an older macrocyst from which the discrete endocytes have disappeared is similarly crushed, the enveloping wall breaks in a comparable manner, but the entire content flows out as a structureless fluid containing innumerable fine particles that immediately exhibit brownian movement as they enter a more aqueous environment.

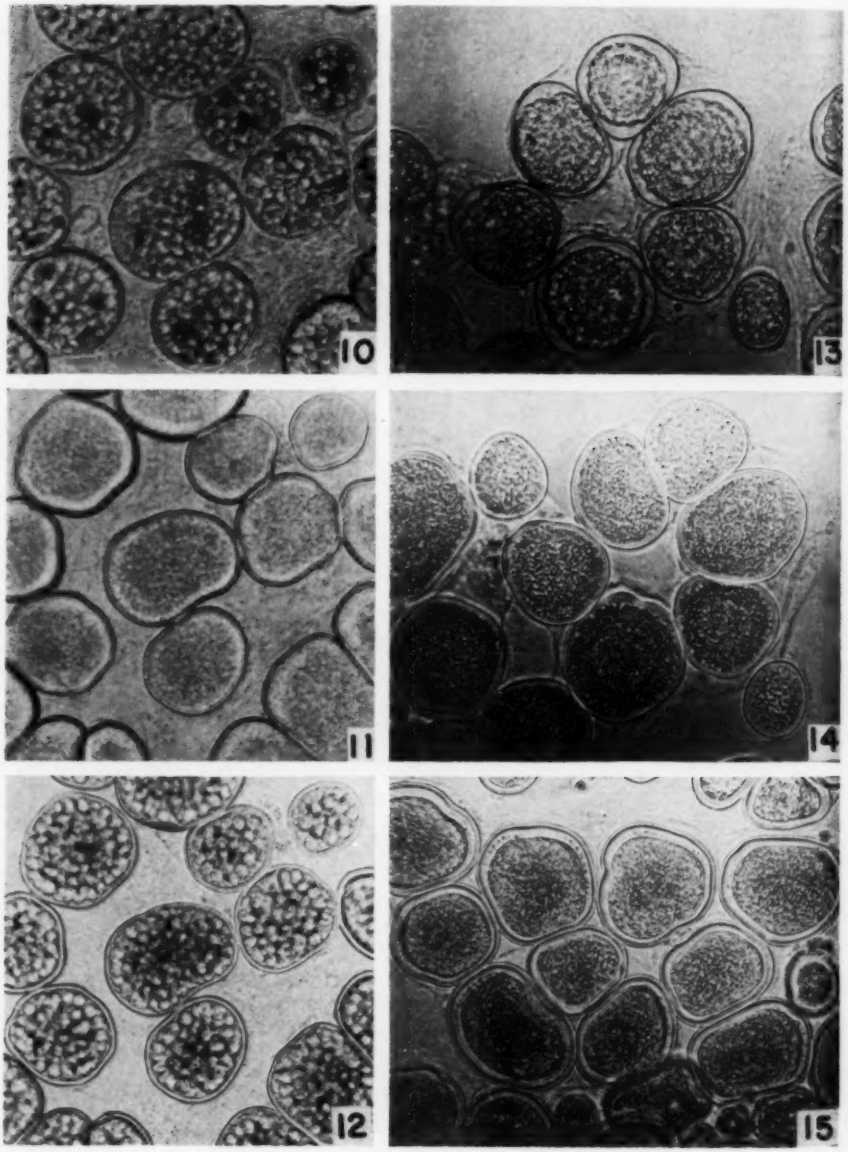
The explanation for endocyte disappearance in naturally aged macrocysts remains unknown, but a superficially similar state can be produced artificially with alkaline solutions. This was first observed when Schweitzer's reagent was applied to preparations of young macrocysts. Upon contact with the cuprammonium solution, the refractive walls of the endocytes disappeared, the seemingly merged content of the entire macrocyst swelled, and with the partial dissolution of the enveloping cellulose wall, the content emerged as a homogeneous and finely granular mass superficially resembling a large and completely undifferentiated protoplast (Fig. 17). A comparable disappearance of endocytes was subsequently observed in preparations following the application of NaOH (1.0 and 0.1 N), albeit the heavy cyst walls remained intact (Figs. 10–11). The application of HCl (1.0 N) to the same preparations prompted a most dramatic phenomenon, for upon contact with this reagent the endocytes reappeared in their original numbers and positions (Fig. 12). The same phenomenon of endocyte reversal accompanied the application of acid following treatment of young macrocysts with Schweitzer's reagent. If the exposed macrocyst was quite young the endocytes emerged individually as the cellulose wall dissolved, and these collected into a spreading amorphous mass within which cell boundaries remained faintly evident, as seen in Figure 16. If the cyst was older (but still packed with endocytes) the content emerged as an intact, seemingly homogeneous mass following similar treatment (Fig. 17). In each case a discrete cellular structure reappeared with the addition of acid (Fig. 18).

Macrocyst germination has been observed with the emergence of amoeboid cells from the ruptured cyst, and it was first thought that such induced disappearance

primary envelope, showing clusters of endocytes surrounded by undifferentiated myxamoebae. × 800.

FIGURE 9. A body comparable to that shown in Figure 8, compressed to release endocytes and still undifferentiated cells. × 800.

PLATE III. Behavior of young (endocyte-filled) and aged (homogeneous) macrocysts in *D. mucoroides*, Strain S-28b, in the presence of alkali and acid.



of endocytes might be analogous to the natural phenomena of macrocyst maturation and germination. However, further experiments failed to substantiate this view, for endocyte reversals did not occur at pH levels that permit growth and other vital activities of the slime mold. Furthermore, in the alternate presence of acid and alkaline solutions an initially endocyte-filled macrocyst could be interconverted repeatedly from an obviously cellular to a seemingly homogeneous state. Such reversal undoubtedly represents a physico-chemical reaction rather than a vital phenomenon, since macrocysts pre-killed with iodine-alcohol or Schaudinn's fixative react as do untreated structures.

The aforementioned tests in combination with certain others have provided substantial information regarding the wall structure of the macrocysts, and specimens treated alternately with alkali and acid and stained with chloroiodide of zinc, followed by Schweitzer's reagent, have proved particularly revealing. The "wall" of a mature, endocyte-filled macrocyst is seen to consist of three strikingly different parts: (1) an outer, loosely fitting primary covering of indeterminate form which characteristically surrounds one or more macrocysts, and may be continuous with, or adherent to, comparable envelopes of adjacent structures (Figs. 24–26); (2) a very much thicker secondary layer that is uniform in thickness, smooth in contour, and usually circular or ellipsoidal in outline (Fig. 26); and (3) an inner membrane formed by the endocytes prior to their disappearance as discrete cellular entities (Fig. 22). Both the primary covering and the secondary wall contain cellulose, apparently embedded within a matrix of more resistant material (Fig. 24–25). Both stain blue in chloroiodide of zinc and both are birefringent when viewed with polarized light, yet neither is completely dissolved by Schweitzer's reagent. However, each loses its birefringence upon the addition of the cuprammonium or an aqueous solution of 72% H_2SO_4. The primary membrane contains relatively little cellulose, embedded within a mucus-like material, and is reminiscent of the slime track and the slime sheath seen in *D. discoideum;* in contrast, cellulose constitutes the principal building substance of the thick secondary wall (Figs. 23 and 29), just as it does in the sorophore sheath of *Dictyostelium* (Raper and Fennell, 1952). The tertiary wall, if it may be so designated, contains no cellulose and represents a continuous film formed in a peripheral position by the mass of differentiating endocytes. This thin, innermost layer is non-rigid and contracts or expands with changes in the volume and character of the protoplasmic material that

PLATE III

FIGURE 10. Ten-day-old macrocysts produced on 0.1 L–P medium containing Nile blue sulfate, untreated. × 360.

FIGURE 11. The same macrocysts following exposure to 1.0 N NaOH; note that all evidence of the constituent endocytes has disappeared. × 360.

FIGURE 12. The same macrocysts following the application of 1.0 N HCl; note how the endocytes have reappeared. × 360.

FIGURE 13. Six-week-old macrocysts showing the typical contracted homogeneous content of such structures, untreated. × 360.

FIGURE 14. The same macrocysts following exposure to 1.0 N NaOH; note how the contents have swelled. × 360.

FIGURE 15. The same macrocysts, displaced in their relative positions, following the application of acid; note that the cyst contents have contracted somewhat, but that they show no evidence of a cellular structure. × 360.

PLATE IV. Behavior of young and aged macrocysts in Schweitzer's reagent.

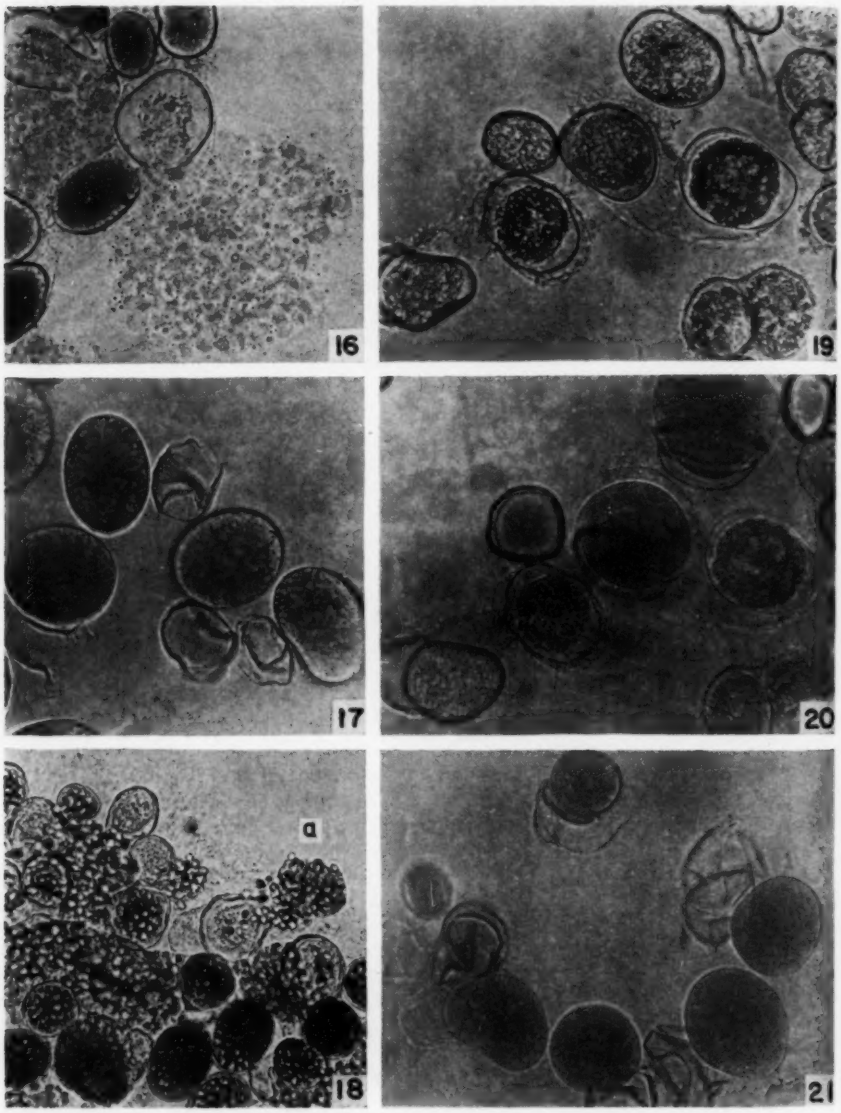


FIGURE 16. Young (endocyte-filled) macrocysts following exposure to Schweitzer's reagent, showing how the cuprammonium solution dissolves the cellulose walls of the macrocysts allowing the endocytes, faintly discernible, to escape. × 360.

it envelops, functioning as the tenacious covering that confines the merged cytoplasmic content of the aged macrocyst when this is released by dissolution of the heavy cellulose wall (Fig. 21). In contrast, when an aged but untreated cyst is broken by mechanical pressure this covering obviously ruptures with the cellulose wall which surrounds it (see above).

Similar preparations afforded equally interesting if more perplexing observations concerning the behavior of the contracted acellular protoplasmic masses present in the older macrocysts. When exposed to NaOH the shrunken content lost the brownish color characteristic of aged cysts and swelled to fill completely the heavy macrocyst wall. Upon the substitution of acid, the brownish pigmentation returned in part and the content again contracted, but not to its former dimensions (Figs. 13–15). Treated with Schweitzer's reagent the content swelled once more and erupted from the disintegrating thick cellulose wall as a single, seemingly homogeneous mass. Significantly, no return to a cellular structure has been observed in the content of any older cysts treated with cuprammonium or NaOH solutions upon the addition of acid (Fig. 15). Thus, there is evidence that the endocytes actually disappear, either by fusion or disintegration, at the time the aging macrocyst assumes a homogeneous appearance. But why does the emergent content remain intact following treatment with cuprammonium (Fig. 21) instead of flowing out freely as when the wall of the untreated homogeneous macrocyst is broken? Does the protoplasmic content represent, in fact, a plasmodium-like mass formed by the fusion of endocytes? Possibly so. Does the macrocyst wall actually consist of three layers, the innermost persisting about the freed protoplasmic body, not because of its inherently greater strength but because it is cuprammonium-resistant? Possibly this is true, for upon the application of slight pressure this bounding membrane breaks, permitting the fine granular content to escape whilst the membrane *per se* remains as a delicate, irregularly wrinkled and contracted envelope exhibiting no birefringence. Alternatively, and more plausibly, if aged macrocysts remain viable, as observations indicate, the thin hyaline envelope thus demonstrated may, in the living state, represent only the inconspicuous and functionally modified protoplasmic membrane of the contracted central body itself.

Two lines of evidence point to the presence of such a semipermeable membrane at progressive stages in macrocyst development. When an endocyte-filled macrocyst is exposed to a saturated solution of NaCl or sucrose, the content as a whole appears to become plasmolyzed and to withdraw from the surrounding thick cellulose wall as seen in Figure 22, plainly demonstrating the presence of a continuous

FIGURE 17. Older macrocysts (but still endocyte-filled) following exposure to this reagent; note how the nearly homogeneous cyst contents remain intact, and how the formerly heavy macrocyst walls are shrunken following the dissolution of their cellulose content. × 360.

FIGURE 18. Macrocysts of the same age as those shown in Figure 16, treated with Schweitzer's reagent and then exposed to 1.0 N HCl; note how the endocytes have reappeared, even in the freed contents of a macrocyst (a). × 250.

FIGURE 19. Two-month-old macrocysts, untreated. × 360.

FIGURE 20. The same macrocysts following treatment with Schweitzer's reagent; note how the outer cellulose wall is being dissolved and how the membrane-encased content is escaping intact. × 360.

FIGURE 21. The same preparation after an additional 10 minutes, showing the intact cyst contents completely free of the macrocyst walls; the latter are no longer birefringent when viewed with polarized light. × 360.

PLATE V. Macrocyst structure.

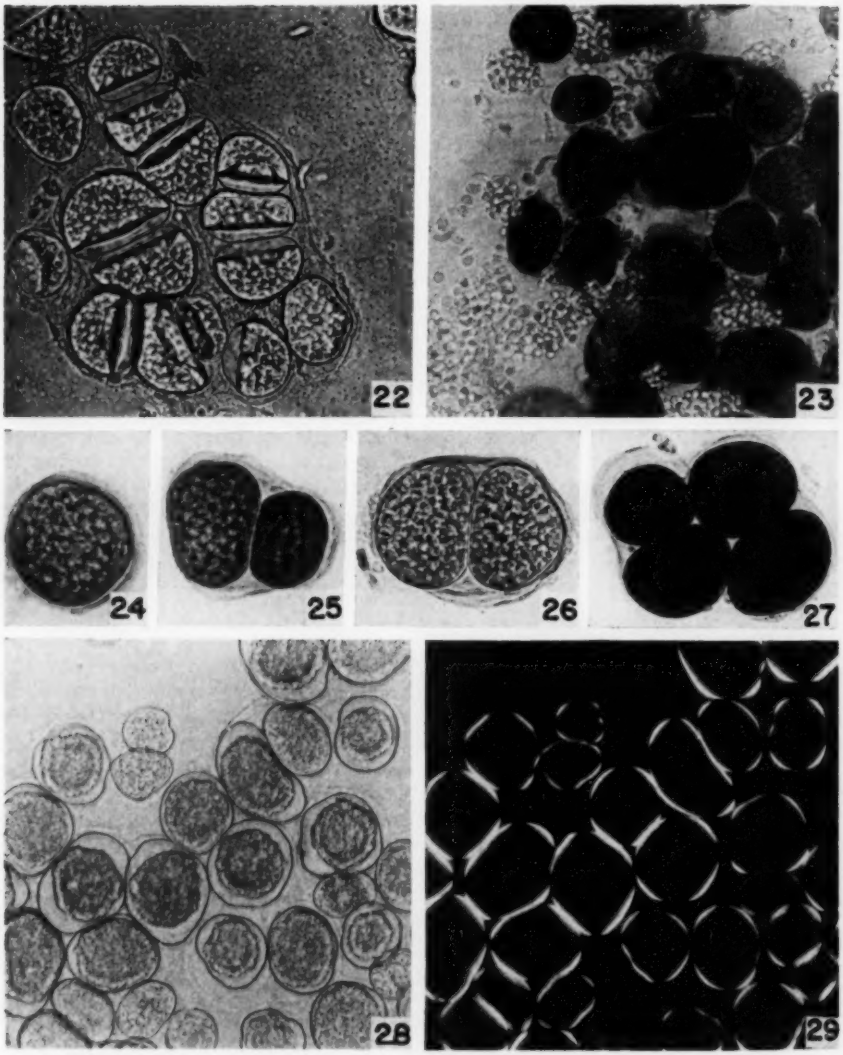


FIGURE 22. Young (endocyte-filled) macrocysts in the presence of a concentrated sucrose solution; note how a membrane, the "tertiary wall," surrounds the endocytes and in the process of plasmolysis pulls away from the rigid cellulose wall which is external to it. × 360.

FIGURE 23. Macrocysts of comparable age stained with chloroiodide of zinc and then subjected to pressure to release the endocytes. × 300.

and differentially permeable membrane external to the constituent endocytes but internal to the heavy cellulose wall. The same unitary pattern of plasmolysis is seen in macrocysts recently turned homogeneous, and in these there is no suggestion of persistent identity for the contributory myxamoebae or endocytes as one might expect if they remained as indistinguishable but nonetheless discrete cells. Aged macrocysts likewise provide contributory evidence. When placed in relatively large volumes of distilled water such structures show variable response depending upon the temperature of incubation. In preparations held at 10° C. evident swelling of the contracted homogeneous content is observed within 72 hours, and after 10 days many empty macrocyst cases, together with abundant free myxamoebae, may be observed (Figs. 41–42). At 15° C. little swelling occurs, even after 10 days, and only an occasional empty case may be seen. At 20 to 25° C. these responses are almost completely lacking (Fig. 40). This behavior is interpreted to indicate a selective permeability mediated by a low and favorable incubation temperature, and it is most unlikely that this could be attributable to the more conspicuous cellulose wall. The evidence would seem to point, indisputably, to the membrane that surrounds the shrunken cyst content.

The formation of macrocysts is observed not infrequently in *Dictyostelium minutum*, but their occurrence in strains diagnosed as *D. mucoroides* is relatively rare. In fact, not more than a half-dozen such isolates have been encountered among the hundreds of strains of the latter species that we have examined. In view of this, it is surprising and noteworthy that Brefeld (1869), in his description of *D. mucoroides*, reported objects believed to be similar to the macrocysts described above. His cultures were grown on microscope slides, and these structures developed in older preparations of that type. He described the structures as "dwarfed sporangia," since at times there was evidence of a rudimentary stalk in the "spore-forming plasm." He reported this type of sporangium to be enclosed by a comparatively thick membrane of cellulose, which upon examination was observed to be stratified and was stained violet with chloroiodide of zinc. This account agrees well with our observations of macrocysts submitted to various tests. He made no mention of the germination of "spores" from the "dwarfed sporangia." Rudimentary stalks such as Brefeld described and illustrated (Fig. 3) have not been observed during these investigations, and we believe that he may have observed immature macrocysts and interpreted clusters of differentiating endocytes as representing rudimentary stalks. In our experience, endocytes first appear in a localized central position within the developing macrocyst, but occasionally such cells do extend to the periphery along a particular radius before comparable differentiation occurs throughout the macrocyst. However, we must not overlook the possibility that, in his particular isolate cultivated under different conditions, he may have encountered stages truly transitional between macrocysts and well-formed sorocarps comprised of sorophores and sori. Certain evidence points to such a possibility. The basic similarity of the aggregative processes leading to sorocarp

FIGURES 24–27. Selected preparations stained with chloroiodide of zinc to show the loosely fitting primary membranes within which 1, 2 or 4 macrocysts have developed; the bodies shown in Figure 27 are older, hence the heavy macrocyst walls stain darkly. × 360.

FIGURE 28. Two-month-old macrocysts as viewed with normal light. × 275.

FIGURE 29. The same as seen under polarized light. × 275.

PLATE VI. Influence of substrates and incubation temperature upon macrocyst formation in *D. mucoroides*, Strain S-28b.

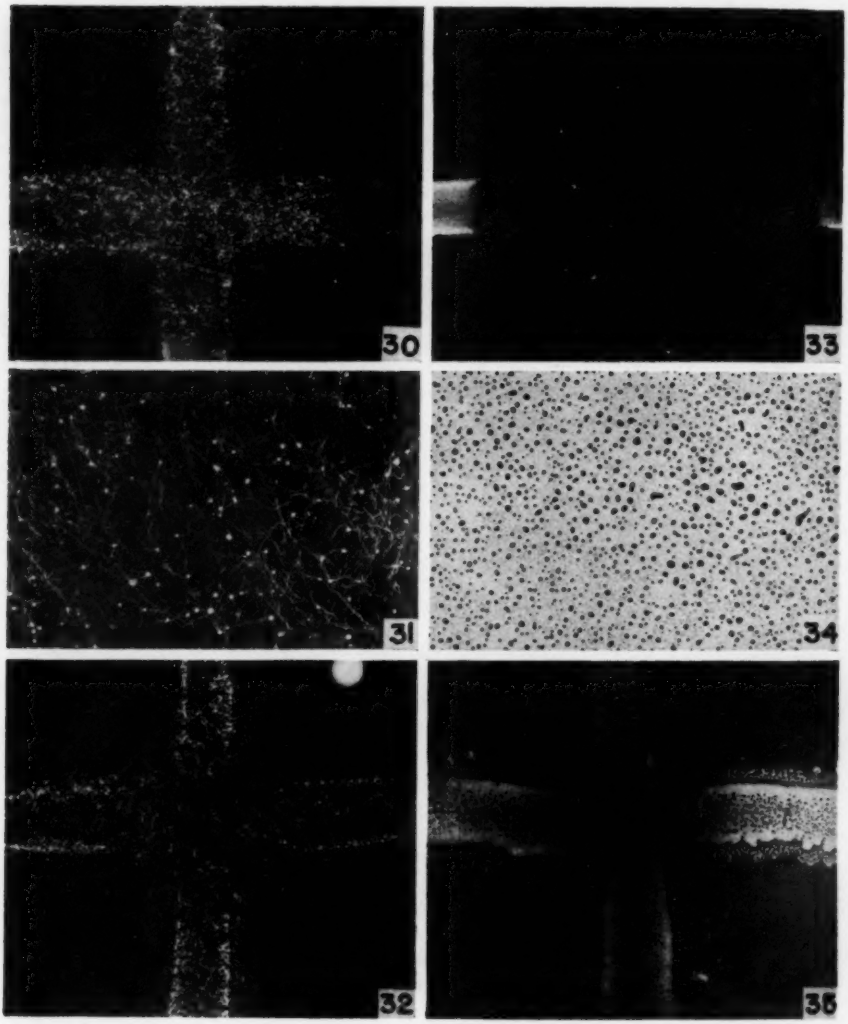


FIGURE 30. "Spore-forming clone" grown in association with *E. coli* on 0.1 L-P medium at 20° C. × 2.

FIGURE 31. Enlarged view of a portion of this culture showing abundant sorocarps and a complete absence of macrocysts. × 5.

FIGURE 32. The same culture as seen in Figure 30, but growing upon a medium containing 0.1% glucose-0.1% yeast extract, at 20° C. × 2.

formation, on the one hand, and to macrocysts on the other is indeed striking, and we have commonly observed in a single microscopic field separate pseudoplasmodia undergoing differentiation in these two directions simultaneously. Such a situation is illustrated in Figure 36. Unfortunately, we have no adequate explanation of the subtle differences that underlie this contrasting behavior, but it is easily conceivable that one might encounter individual cases where the shift from one to the other of these morphogenetic processes would be incomplete, as suggested by some of Brefeld's illustrations.

The formation of macrocysts may in effect represent an aberration of the normal fruiting process, or it may represent an alternative pattern of differentiation with implications of far-reaching significance that are yet unappreciated. The heavy cellulose wall which it develops bears in many ways a striking resemblance to the sorophore sheath, so essential to the construction of the normal sorocarp (Raper and Fennell, 1952), but with this important distinction: the wall of the macrocyst is secreted external to the whole mass of myxamoebae that contribute to the formation of this body, whereas the sorophore sheath is produced at a critical circular locus within the mass by a limited group of specialized cells which subsequently differentiate as the vacuolate cellular elements of the sorophore itself.

We have, at present, an incomplete picture of the morphology of the macrocyst. We have convincing evidence that they can germinate and re-initiate the life cycle of the slime mold. However, we do not know the fate of the cells which enter the macrocyst, and we do not know the origin of those which subsequently emerge. Until such information is at hand we cannot compare in any definitive sense the morphogenetic processes that underlie these contrasting developmental stages. A thorough cytological and histological study is clearly needed and will be undertaken at the earliest possible opportunity.

Factors influencing the formation of macrocysts

The obvious factor most directly affecting macrocyst formation in *Dictyostelium mucoroides* and *D. minutum* is the inherent genetic constitution of the particular isolate. Within our experience, a minority of *D. minutum* strains and only an occasional isolate of *D. mucoroides* have exhibited this capacity. However, once this ability has been demonstrated for a culture, it is sometimes possible to alter markedly the relative proportions of macrocysts and of normal sorocarps by changing the conditions under which the slime mold is cultivated. The responses of *D. mucoroides,* strain S–28b, have been studied in considerable detail and the observations subsequently recorded apply particularly to that strain. Whereas the various factors that influence the ratios of sorocarps to macrocysts are invariably interrelated, certain conditions which strongly affect these balances have been identified and investigated more or less independently.

Temperature: Second only to genetic constitution is the influence of the incubation temperature. When cultivated at 24–25° C. in association with *Escherichia*

FIGURE 33. "Cyst-forming clone" growing in association with *E. coli* on 0.1 L–P medium at 20° C. × 2.

FIGURE 34. Enlarged view of a portion of this culture showing abundant macrocysts and a complete absence of sorocarps. × 7.5.

FIGURE 35. The same culture as seen in Figure 33, but growing upon a medium containing 0.1% glucose-0.1% yeast extract at 20° C. × 2.

PLATE VII. Macrocyst formation in *Dictyostelium mucoroides*, Strain WS-47.

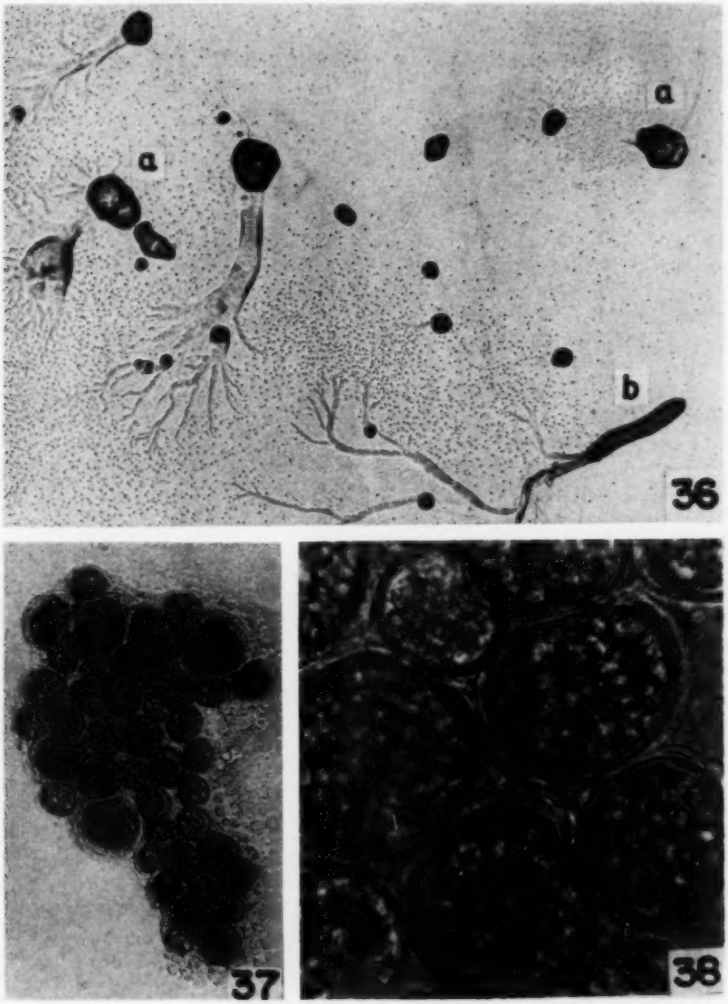


FIGURE 36. Radiate pseudoplasmodia leading to macrocyst formation (a) and to sorocarp formation (b) within the same microscopic field, as seen with low magnification. × 24.

FIGURE 37. Irregular clump of macrocysts developing from a single pseudoplasmodium such as that shown in the preceding figure. × 125.

FIGURE 38. Detail of some of the macrocysts seen in Figure 37. × 600.

PLATE VIII. Macrocyst germination in *Dictyostelium mucoroides*, Strain S-28b.

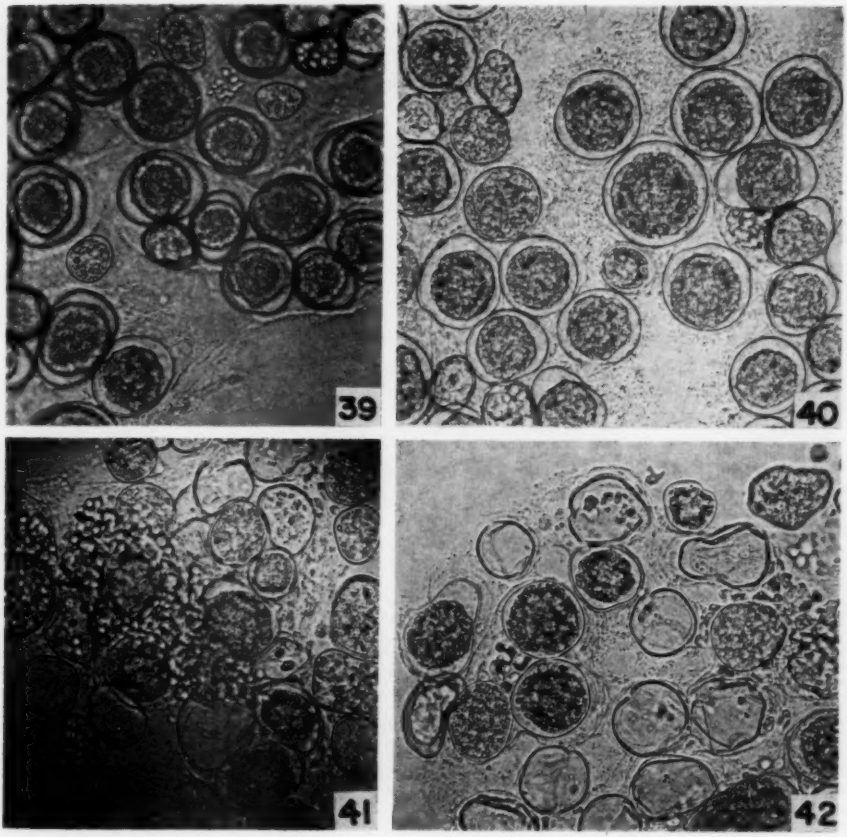


FIGURE 39. Two-month-old macrocysts incubated continuously at 20° C. and removed from an agar plate immediately before being photographed; note the single macrocyst at upper right which still contains endocytes. × 300.

FIGURE 40. Macrocysts similar to the preceding, but removed from agar and incubated in distilled water at 20° C. for 10 days prior to photography; note that little evident change has taken place. × 300.

FIGURE 41. Macrocysts like the preceding, but incubated for 10 days at 10° C. just prior to being photographed; note the empty cyst cases and the mass of free myxamoebae which have escaped from these. × 300.

FIGURE 42. Photomicrograph showing a second field from the same preparation shown in Figure 41; note the broken walls of the empty cyst cases, also that a few myxamoebae still remain within the germinating cyst at top center. × 300.

coli on 0.1 L–P, hay-infusion or thin hay-infusion agars, the stock strain of S–28b regularly produces both sorocarps and macrocysts in abundance, although individual cultures and even different areas within the same Petri dish commonly exhibit conspicuously disproportionate ratios of these contrasting structures. If the temperature is raised as little as two or three degrees the ratio of macrocysts to sorocarps is increased substantially; conversely, if it is lowered to 20° C. or less this ratio is strongly depressed.

Clonal substrains of S–28b have been isolated which exhibit temperature sensitivity even more dramatically. These were obtained by heating spore suspensions in standard salt solution (Bonner, 1947) for varying lengths of time and then, following appropriate dilution, plating the spores in association with *Escherichia coli* on 0.1 L–P agar. Plaques, evidenced by clearance of the bacteria, developed after four days in plates incubated at 20° C., presumably from single spores. Some of these subsequently developed only macrocysts, others produced only sorocarps. By re-isolation from such contrasting areas, a "cyst-forming clone" and a "spore-forming clone," with strikingly different temperature responses, were isolated (Figs. 30–35). Upon continued recultivation on 0.1 L–P agar, the former characteristically produced only macrocysts at 20° C. but developed abundant sorocarps and scattered macrocysts at 15° C., whereas the latter typically produced only sorocarps at 20° C. but formed both sorocarps and macrocysts at 25° C. Cultures initiated with spores from the cyst-forming clone grown at 15° C. developed macrocysts when recultivated at 20° C., demonstrating a marked degree of genetic specificity in the myxamoebae and spores of the clone. Thus the pattern of cellular differentiation exhibited by different clones of S–28b results from inherited characteristics that are temperature-dependent for their expression.

Culture media: The production of macrocysts is strongly influenced by the substrates upon which the slime mold is cultivated, and these structures are regularly formed in greater abundance upon 0.1 L–P agar than upon media based upon hay-infusion. Their development is even further accentuated if yeast extract is substituted for peptone, this being used either as the sole nutrient or in combination with lactose or glucose. For example, upon 0.1% lactose-yeast extract or 0.1% glucose-yeast extract agars abundant macrocysts are formed by the cyst-forming clone incubated at 15° C. and by the spore-forming clone at 20° C., in each case at temperatures where few or no macrocysts normally develop on 0.1 L–P agar. An intimate association obviously exists between substrate composition and incubation temperatures as these factors affect macrocyst formation, but just how they condition the cultural environment and how they affect the fructifying myxamoebae, influencing them to produce either macrocysts or sorocarps, has not been determined (Figs. 30–35).

Bacterial associate: Dictyostelium mucoroides, S–28b, can be cultivated successfully with a variety of bacterial associates on the nutrient-poor media employed in this investigation. Gram-negative bacteria support better growth of the slime mold than do Gram-positive types, and of the former *Escherichia coli* was the most favorable species investigated. *Aerobacter aerogenes, Pseudomonas fluorescens* and *Serratia marcescens* yielded satisfactory but less luxuriant slime mold growth. The formation of macrocysts and sorocarps followed, in general, the patterns observed with *E. coli.* The myxamoebae of strain S–28b digest or destroy the red pigment (prodigiosin) of *S. marcescens,* hence yield uncolored sorocarps and

macrocysts. In marked contrast, the cells of strain NC–12, like those of *D. discoideum*, retain the pigment (Raper, 1937), hence produce sorocarps and macrocysts that are pink in color. *Bacillus subtilis*, a Gram-positive bacterium, supported fair growth of the slime mold and the formation of abundant macrocysts on 0.1% lactose-yeast extract agar at 20° C. *Sarcina lutea*, a second Gram-positive species, permitted only limited growth of myxamoebae and few macrocysts were produced on any substrate. An experiment conducted to determine what effect the age of the nutritive *E. coli* might have on growth and macrocyst formation in *D. mucoroides* failed to reveal any significant differences.

Hydrogen-ion concentration: A heavy suspension of *E. coli* cells previously grown in 0.1 L–P broth and concentrated by centrifugation was cross-streaked on 0.1 L–P agar plates to investigate the effect of pH on slime mold growth and macrocyst formation. The underlying agar substrate was adjusted over a range of pH from 3.3 to 9.2 using the buffers previously cited. In these experiments growth of myxamoebae was obtained between pH 4.5 and 8.0. Slight evidence of aggregation was observed at pH 4.5 but no well-defined pseudoplasmodia developed, and of course no sorocarps or macrocysts were produced. A heavy development of sorocarps and/or macrocysts was obtained between pH 5.5 and 7.0, depending upon the incubation temperature. No growth occurred above pH 8.0 where K_3BO_3 was used as buffer. Questioning whether it was the high pH or the borate buffer which inhibited growth, we made additional tests using KH_2PO_4 as buffer with the addition of NaOH to yield pH levels from 7.5 to 9.0. Growth of the stock culture occurred to pH 9.0, but few and abnormal sorocarps and no macrocysts were formed. Even in the cyst-forming clone incubated at 20° C. where one would normally expect only macrocysts, many sorocarps and very few macrocysts developed on the more alkaline substrates. The pH of the medium did not change appreciably during the period of slime mold growth.

Ammonia concentration: Cohen (1953) reported that ammonia suppressed normal morphogenetic development in the Acrasieae, either inhibiting growth completely, or causing various abnormalities in sorocarp formation. His procedures were carefully followed to ascertain whether different concentrations of ammonia would enhance macrocyst formation. It was thought that if these structures represented aberrant sorocarps, their production might be enhanced under conditions where sorocarp formation was inhibited. However, macrocysts were not formed in any of the ammonia concentrations employed, possibly indicating a suppressive effect comparable to that reported for sorocarps by Cohen.

Relative humidity: The effect of per cent relative humidity on slime mold growth and the development of macrocysts was tested by the procedures previously indicated. Large depression and ordinary flat slides with one ml. of 0.1 L–P agar were employed, rather than Petri dishes, to insure rapid establishment of an equilibrium between the moisture of the agar and the atmosphere in the desiccators. Olive (1902) attributed the encystment of myxamoebae (microcysts) to adverse conditions such as the drying of culture substrates. If macrocyst formation should represent a stage comparable to the encystment of individual myxamoebae, we would have anticipated more macrocysts when the per cent relative humidity was lowered, causing a drying of the substrate. Such did not occur in our tests.

Macrocyst formation in other strains: Attempts were made to enhance macrocyst formation in other strains of *D. mucoroides* (WS–47 and NC–12) and in *D.*

minutum (WS–56–2 and Purdue 8a). Cultural conditions were varied much as previously outlined for *D. mucoroides*, S–28b. However, in these additional slime molds neither the type of culture medium nor the concentration of its ingredients seemed to markedly affect the relative number of macrocysts produced. For example, the proportion of macrocysts to sorocarps remained relatively constant upon media containing yeast extract *vs.* peptone and upon nutrient-rich *vs.* less concentrated media, although total growth of the bacteria and of the slime mold varied substantially with such changes.

A variety of culture media were buffered and adjusted between pH 6.0 and 8.5. All supported growth of the slime mold, and in no case was there an exceptional increase in macrocyst production.

Temperature relationships were examined carefully. *Dictyostelium mucoroides*, WS–47 (Figs. 36–38), produced more macrocysts at 20° and 25° C. than at 15° C., but this response was not so striking as in strain S–28b. A few macrocysts developed in strain NC–12 at 20° C., but at 25° C. only irregular growth of the slime mold occurred, indicating too high an incubation temperature. In *D. minutum*, Purdue 8a, approximately equal macrocyst formation was observed at 20°, 25° and 28° C. In contrast, WS–56–2 formed no macrocysts at 20° C., only a very few at 25° C., and grew very poorly at 28° C.

Germination of macrocysts

The possible role of the macrocysts in the life cycle of *Dictyostelium* engaged our attention from the outset, since it did not seem reasonable that such structures produced in great abundance and under seemingly optimal conditions would represent a terminal and functionless kind of differentiation. For this reason much thought and effort have been given to their germination. In this, as in other phases of the investigation, studies have been centered upon *D. mucoroides*, S–28b, and our researches have been greatly facilitated by the cyst-forming clones. By capitalizing upon their unique temperature responses it has been possible to produce at will large populations of macrocysts under cultural conditions where no spores were formed. At the same time any vegetative myxamoebae which might have adhered to the macrocysts were readily killed by heating at 42° C. for 10 minutes. No growth of the slime mold occurred when the macrocysts were heated at a temperature high enough to kill the mature spores, and such would have been present in any macrocyst preparations taken from the parent culture. The comparative temperature tolerances of the myxamoebae and spores of *D. mucoroides*, S–28b, are shown in Tables I and II, respectively.

Proceeding on the assumption that the macrocysts might represent a resistant stage in the cycle of *Dictyostelium*, they were subjected to a variety of cultural environments and treatments. Plates of 0.1 L–P agar containing abundant macrocysts were alternately frozen at − 10° C. and thawed at + 25° C. in an attempt to instigate germination, but consistent results were not obtained. Growth from macrocysts treated in this way was twice observed after ten days' incubation. Crump (1950) had reported that raising the temperature favored the germination of encysted free-living amoebae, but no germination ensued after heating macrocysts in a water bath for five minutes at 55° C.

At the suggestion of the late Dr. Charles Thom, an attempt was made to stimu-

late macrocyst germination by spreading a heavy inoculum of pre-grown cysts and *E. coli* on sterilized soil in Petri dishes and incubating the plates at 10°, 15° and 20° C. No evidence of germination was obtained in 28 days, although we had demonstrated previously that growth of the slime mold could take place under these natural conditions.

Brefeld (1869) stated that germination of *Dictyostelium* spores took place only in a nitrogenous medium and suggested fresh horse dung decoction and uric acid media as substrates. Both were investigated. No evidence of macrocyst germination was observed on fresh horse dung agar plates, but growth was obtained, in association with *E. coli*, on 0.05% uric acid agar after eight days incubation at 15° C.

TABLE I

*Temperature tolerance of myxamoebae of Dictyostelium mucoroides, S-28b, suspended in distilled water**

Time in water bath, minutes	Temperature, ° C.	Relative amount of growth per test		
		1	2	3
0	42	++++	++++	++++
5		0	+	+
10		0	0	0
15		0	0	0

++++ = Excellent growth.
+++ = Very good growth.
++ = Good growth.
+ = Limited growth.
0 = No growth.

* Similar results were obtained when myxamoebae were suspended in standard salt solution.

Macrocysts were dismembered in a McShan-Erway tissue homogenizer in the hope that the endocytes thus freed would re-initiate growth under suitable cultural conditions. Such homogenates were mixed with *E. coli* and the resulting suspensions spread on 0.1 L–P agar plates. No growth of the slime mold ensued, but the possibility of serious injury to the endocytes could not be discounted.

Despite the negative results obtained, the seeming logic of this approach led us yet again to attempt the dissolution of the macrocyst wall by other means as a possible aid to germination and regrowth. The procedure employed was probably ineffective *per se*, but in performing the experiment the "treated" macrocysts were incubated at a variety of temperatures which provided a clue to cultural conditions where germination not infrequently occurred. A cellulase preparation, contributed by Dr. Emory G. Simmons, was investigated as a means of digesting the heavy enveloping macrocyst wall. The enzyme was employed as a 1% solution in *M*/20 citrate solution at pH 5.0. Macrocysts were harvested from cultures where no spores had formed, suspended in the cellulase-citrate solution, and heat-treated to kill any adherent myxamoebae. Germination occurred in varying amounts in the cellulase-treated macrocysts and also in controls similarly heat-treated in standard salt solution. The earliest evidence of germination was observed after 6 days'

incubation, and new plaques continued to develop for as long as 22 to 40 days at varied temperatures. The percentage germination was approximately the same for macrocysts heat-treated in the cellulase-citrate and in the standard salt solutions. The earliest evidence and the greatest amount of germination were observed among the macrocysts incubated at 15° C., with decreasing amounts to 25° C. Actual

TABLE II

Temperature tolerance of spores of Dictyostelium mucoroides, S-28b, suspended in standard salt solution (Test 1) and in distilled water (Tests 2, 3, and 4)

Time in water bath, minutes	Temperature, ° C.	Relative amount of growth per test			
		1	2	3	4
0	42	++++			
90		++++			
0	50	++++	++++		
65		+	+		
0	55	++++	++++		
5		++	+++		
10		0	+		
15		+	+		
20		0	+		
25		0	0		
30		0	+		
35		0	0		
0	60	++++	++++	++++	++++
5		+++	++	+	++
10		+	++	+	0
15		0	++	0	0
20		0	+	0	+
25		0	+	0	0
30		0	0	0	0
0	65	++++	++++	++++	
5		+	+	0	
10		0	0	0	
15		+	0	0	
20		0	0	0	

++++ = Excellent growth.
+++ = Very good growth.
++ = Good growth.
\+ = Limited growth.
0 = No growth.

germination of a macrocyst, or of the endocytes contained within it, was not then observed, but in some instances one or more empty macrocyst cases were evident where a plaque of growth occurred.

It was now hoped that even though the percentage viability was apparently low, a few macrocysts might be seen to germinate if these were carefully isolated and observed periodically over a period of several days. Single macrocysts of dif-

ferent ages (3, 15 and 35 days) were selected, heat-treated to kill any vegetative myxamoebae, and placed individually in marked squares on 0.1 L–P agar plates smeared with *E. coli*. Evidence of macrocyst germination was noticed after incubation at 15° C. for five days.

A series of experiments was undertaken to determine whether macrocysts of a particular age would germinate more readily than cysts of other ages; and since presumptive germination had seemed to vary at different incubation temperatures, special consideration was given to this matter. To obtain macrocysts of specific ages, a heavy suspension of myxamoebae of the cyst-forming clone and *E. coli*

TABLE III

Percentage germination among macrocysts of Dictyostelium mucoroides (No. S-28b) of different ages, and the number of days before such germination was observed at different incubation temperatures

Age of macrocysts, days	Temperature of incubation									
	5°		10°		15°		20°		25°	
	Days	%	Days	%	Days	%	Days	%	Days	%
5 (1)*	35	0	35	0	35	0	35	0	35	0
(2)*	44	0	18	0.6	30	0.2	30	0.07	38	0
10 (1)	35	3.4	20	3.1	22	7.2	28	4.5	42	0
(2)	30	+**	14	+	14	3.0	25	0.7	30	0
15 (1)	38	1.6	18	2.9	12	1.2	18	5.5	38	0
(2)	43	0	20	+	20	+	20	+	37	0
20 (1)	25	0.5	18	1.8	12	1.0	12	1.0	32	0.2
(2)	38	0	23	1.0	15	0.9	15	1.0	29	0
25	38	+	15	4.0	15	+	18	1.0	30	0
35	28	+	20	+	8	0.2	8	0.1	20	0.07

* Indicates separate experiments.

** (+) Indicates germination of macrocysts, but percentage of total population could not be calculated.

was spread over the surface of 0.1 L–P plates and incubated at 20° C. for 5, 10, 15, 20, 25 and 35 days. These macrocysts were then harvested, heat-treated to eliminate all vegetative myxamoebae, spread on fresh agar plates with *E. coli*, and incubated at 5°, 10°, 15°, 20° and 25° C. for 5 to 6 weeks. The results of these experiments are summarized in Table III.

Substantial growth of the slime mold was obtained in certain of the above tests, particularly in plates inoculated with 10- and 15-day-old macrocysts incubated at intermediate temperatures, the highest percentage (7.2%) being observed in 10-day cysts incubated at 15° C. The prevalence of empty macrocyst cases in the developing plaques, the prior heat-treatment of the cysts to kill adherent myxamoebae, the carefully prepared and examined source plates from which the macrocysts were

taken for these experiments, and the observed presence of empty macrocyst cases at central locations within many plaques, all convinced us that the observed growth must have developed from germinated macrocysts.

Nevertheless, we had not actually observed this phenomenon, and even in the most consistent macrocyst-forming culture it is possible that a minute sorocarp could go unobserved and that an occasional spore, which would not be killed by heating to 42° C., might be carried over with the implanted macrocyst. An experiment was carried out to determine whether an occasional spore, if present, might have served as the initiator of the plaques of amoeboid growth in the macrocyst germination plates. It was known that growth from spores would eventually occur at all of the incubation temperatures employed (5° to 25° C.); therefore, the times required for plaques to develop from individual spores under cultural conditions duplicating the above were determined. At 20° C. plaques were evident within 4 days, at 15° and 25° C. within 6 days, at 10° C. within 11 days, while at 5° C. growth was not evident until 25 days. Since plaque formation is usually optimal at 15° C. on the macrocyst germination plates, and does not become evident until after 8 to 11 days, these results provided additional evidence that the observed growth resulted from macrocysts and not from occasional contaminating spores.

Having determined the optimum cyst age and the incubation temperature that are favorable for macrocyst germination, 10-day macrocysts in association with *E. coli* were implanted on freshly poured plates and on sterile Maximov slides containing 1.0 ml. of 0.1 L–P agar to observe the germination of the macrocysts directly. Realization of this objective proved unexpectedly time-consuming, but it was accomplished. Germination in this instance, as in the majority of cases observed up to this time, was from a macrocyst filled with endocytes at the time of implantation in the test culture. Pre-germination changes were not observed but it is assumed that the heavy macrocyst wall was ruptured either by swelling of its content and/or by enzymatic dissolution (see below).

Re-examination of Table III reveals that maximum germination occurred among the 10-day-old macrocysts; *i.e.*, structures which were packed with endocytes at the beginning of the tests. More significantly, appreciable germination was recorded for some of the older macrocysts, notably the 25-day cysts incubated at 10° C. This result is especially interesting since cysts of this age would have already lost their discrete endocytes, in the great majority of cases, and would have assumed the homogenous appearance that characterizes aged cysts. Thus presumptive evidence was obtained that macrocysts of the latter type are capable of germination—presumptive because even in preparations taken from cultures after several weeks occasional macrocysts are seen in which the endocytes remain distinct, and the recorded germination could have resulted from such non-homogeneous structures. The improbability of this explanation was subsequently demonstrated. Blocks of 0.1 L–P agar bearing abundant homogeneous macrocysts aged 6 and 8 weeks were placed in sterile Petri dishes, flooded with sterile distilled water, and incubated at 10°, 15°, 20°, and 25° C. Within only 10 days approximately half of the cysts of both ages incubated at 10° C., and only at this temperature, had germinated among populations where prior examination had established that only occasional cysts (2–4%) were still in the endocyte stage at the beginning of the experiment (Figs. 39–42). An understanding of the intracystic events which transpire during the progression from endocyte differentiation to their subsequent disappearance,

and from this through the stage of seeming homogeneity and protoplasmic contraction to the eventual swelling of this mass and the reappearance of amoeboid cells at the time of germination, must await careful cytological investigation. For the present we can only record that in the presence of a proper aqueous environment and at a favorable temperature the previously shrunken "protoplast" (long believed doubtfully viable) swells and gives rise to myxamoebae which escape during cyst germination. Additionally, there is evidence that these myxamoebae, or the parent coenocyte (?), produce cellulolytic enzymes which facilitate rupture of the heavy cyst wall, for viewed with the polarizing microscope the empty cyst cases contain conspicuously less cellulose than do the walls of macrocysts still ungerminated.

Formation and germination of microcysts

Many members of the Acrasieae are characterized by a second, simpler type of encystment stage where individual vegetative myxamoebae round up and become encased by relatively thin protective membranes (Fig. 1). The walls of these resting cells, or microcysts, like those of the macrocysts and the more resistant spores, are predominantly cellulosic in composition. As reported by Olive (1902), there is ample evidence that these form in response to sub-optimal growth conditions. It is probable that the myxamoebae of any member of the Acrasieae may enter such a stage temporarily, but they are most commonly encountered, and in greatest numbers, in isolates of *Dictyostelium minutum, D. polycephalum, Polysphondylium pallidum,* and *Acytostelium leptosomum.*

Microcysts of several species of the Acrasieae were examined to determine their method of germination. Microcysts were placed in hanging-drop slides in thin-hay broth with killed cells of *E. coli* and incubated at 25° C. Within two days many of the microcysts had germinated, as evidenced by the number of free, feeding myxamoebae and by the empty microcyst cases from which these had emerged. Previous workers (Olive, 1902) had not reported true excystment of the microcysts, but had intimated that the myxamoebae absorbed the protective covering, or wall, during germination. The emptied cases are extremely delicate and hyaline, and some reveal a fairly obvious opening at one side through which the myxamoeba escaped. They do not germinate by the emergence of the protoplast through a pore or exit tube, neither do they appear to split as do the spores of most species; rather, germination appears to take place by the dissolution of a fractional portion of the microcyst wall. The cyst cases stain violet-blue with chloroiodide of zinc and show a weak birefringence, indicating that they contain some cellulosic material.

In *Dictyostelium mucoroides* microcysts are about twice the dimensions of the endocytes that comprise the macrocysts, ranging from about 5.0 to 7.5 μ in diameter and being generally spheroidal.

Discussion

Intriguing questions are posed by the macrocysts of *Dictyostelium* with regard to their morphogenesis and their probable primary function in the life-cycle of these slime molds. Do they represent a normal but generally unrevealed stage in the

development history of the Acrasieae, *i.e.*, could they be demonstrated in all members of the group if we but knew the conditions required to evoke them? Do they provide a resting stage whereby these micro-organisms survive otherwise impossible environmental conditions? Do they perhaps constitute some unanticipated manifestation of a sexual stage? Or do they represent, as their superficial appearance might suggest, groups of myxamoebae so thwarted in their normal morphogenesis that they become "captives" doomed to a type of terminal differentiation approximating that of sterile stalk cells? Does the identity of the contributory myxamoebae remain unchanged during the formation of the endocytes, and do the latter in some altered form persist to once again emerge during germination as myxamoebae capable of perpetuating the species? For certain of these questions we have succeeded in providing partial answers.

Brefeld illustrated some "dwarfed sporangia" that contained differentiated cells which he interpreted as representing elements of abortive stalks, and early in this investigation we questioned whether the endocytes might not in fact reflect a type of cellular differentiation of this type. More careful examination has established beyond question that such is not the case despite certain superficial similarities in appearance. The walls of the endocytes contain no demonstrable cellulose and the content of such cells is actually condensed, whereas the walls of true stalk cells contain cellulose and the cell content is strongly vacuolate, occupying a peripheral position within the semi-rigid cell. Upon treatment with alkali (*e.g.*, 1.0 N NaOH) the walls of stalk cells do not disappear as do those of the endocytes.

Much evidence supports the belief that macrocysts arise through an orderly and natural morphogenetic process, and hence in no wise represent aberrant fruiting structures. For those strains which produce them, they would appear to be no less normal than the sorocarps which regularly develop under similar or, in some instances, altered conditions. A measure of homology is suggested by the basically similar aggregative process which precedes the formation of both types of structure. The pseudoplasmodia leading to macrocyst formation are generally diminutive, but this condition is not a necessary precedent to their formation. Additionally, the myxamoebae entering a pseudoplasmodium destined to form macrocysts rarely show the marked elongation characteristic of cells entering larger aggregations, but this weak cellular response is believed to indicate degree rather than difference, *i.e.*, to reflect a feeble aggregative stimulus incident to, or responsible for, the small pseudoplasmodium.

A point of similarity should be noted between the morphogenetic processes leading to the formation of gregarious sorocarps in certain species (*e.g.*, *D. minutum* and *D. lacteum*) and to clustered macrocysts in *D. mucoroides*, for in both situations the magnitude of the initial pseudoplasmodium often exceeds the number of myxamoebae that can effectively collaborate in producing a single sorocarp or macrocyst. In the former instance, secondary centers appear soon after the overall pattern of the wheel-like aggregate becomes evident, and from each of these a separate sorocarp subsequently develops; in the latter case, multiple loci of endocyte formation similarly appear within the initial aggregate, and from each of these later develops a discrete and typical macrocyst.

Substantial differences characterize subsequent steps in the two morphogenetic processes, and there is little if any evidence to suggest that the macrocysts represent modified or abortive sorocarps. The latter can be produced in any known species

by a variety of devices (*e.g.*, unfavorable pH, increased temperature, etc.), but in no observed instance have such abnormal fruits presented a pattern which is remotely suggestive of macrocyts. Rather, they assume the form of *Guttulina*-like fructifications wherein the myxamoebae produce irregular mounds and undergo incomplete differentiation, but they never form a common protective wall about the mass of cells so assembled.

The heavy cellulosic wall of the macrocyst bears a structural likeness to the sorophore sheath of the normal sorocarp, but, as noted earlier, the relative positions at which these are deposited by the constitutive cells are quite different. Furthermore, the formation of the sorophore sheath is antecedent to cellular differentiation in sorocarp building, whereas it lags behind this phenomenon in macrocyst construction. There is yet another difference which may prove highly significant. Bonner *et al.* (1956) demonstrated that the sorophore sheath is secreted by an ever-changing epithelium-like layer of myxamoebae that are oriented perpendicular to the surface of the wall being deposited, whilst in the macrocysts the last remaining amoeboid cells, hence those adjacent to the developing wall, are oriented in quite the opposite direction. Only in those cultures where seemingly identical and intermixed pseudoplasmodia give rise either to sorocarps or to macrocysts, as in strain WS–47, do we find evidence that the two morphogenetic pathways may be closely allied, and in these we have at present no concept of what major or minor organizational differences may underlie such divergence. Judging from Brefeld's illustrations (1869), it is possible that he may have seen so-called "dwarfed sporangia" that were transitional between sorocarps and macrocysts, but no structures of this type have been observed in our cultures. Finally, we would reiterate that the body of evidence presently available points to macrocyst production as representing an alternative but thoroughly normal morphogenetic pathway that is an inherited character possessed by occasional strains of *D. mucoroides* and by many isolates of *D. minutum*. The isolation of contrasting "cyst-forming" and "spore-forming" clones in strain S–28b strengthens this belief, as does also our inability thus far to induce macrocyst formation in any culture of *D. mucoroides* which did not naturally exhibit this capacity at the time of its isolation.

In contrast to this situation, the capacity to produce microcysts seems to be generally present among the Acrasieae, and it is suspected that every isolate may, under certain variable environmental conditions, exhibit such a resting stage. It should be recognized, however, that this phenomenon is probably totally unrelated to macrocyst formation. Microcysts represent the responses of single myxamoebae to effect a transitory resting stage in the vegetative phase of these slime molds and is perhaps strictly comparable to the encystment of certain small, free-living amoebae. Their natural function is indisputably one of enabling the species to survive otherwise unfavorable environments. The macrocysts, on the other hand, arise through multicellular integration and differentiation and represent the product of a specific morphogenetic process, just as do the sorocarps. This function is still incompletely known.

We have obtained convincing evidence that macrocysts germinate under certain circumstances, emitting amoeboid cells which then re-initiate vegetative growth. But we cannot say with confidence that the macrocysts represent a vital resting stage, as their appearance might suggest. Heat tolerance tests indicate that they can withstand appreciably higher temperatures than vegetative myxamoebae, but

they are in turn less resistant than true spores. While the matter has not been explored under conditions that exist in nature, it is possible that they might be produced under circumstances which would preclude the formation of sorocarps and spores, *e.g.*, in strains such as S–28b at elevated temperatures. The general application of this premise is doubtful, however, for many macrocyst-producing strains fail to show a comparable response. Based upon laboratory tests, we could not, at present, conclude that they possess singular survival value.

Possibly they are endowed with other unique properties, a suggestion presently based less upon fact than fancy. We find it difficult to dismiss lightly a structure of multicellular origin which appears to be so highly organized as the older macrocyst. We cannot say with absolute certainty that its content represents a single homogeneous multinucleate protoplast, but such tests as we have applied would seem to support this notion. If the endocytes do actually lose their identity, as appears to be the case, the acellular content of the aged macrocyst would represent a coenocyte, or to use a term more commonly associated with slime molds, perhaps a plasmodium, albeit one that is enclosed within a heavy cellulose wall. Such a plasmodium would of course be quite unlike that which Brefeld (1869) once thought to be present, or that which Skupienski (1920) envisioned as an accompaniment to reported sexuality in *Dictyostelium*. Needless to say, it would represent quite a different structure from the large vegetative body that occupies so conspicuous a place in the life-cycle of the Myogastrales. Clearly, the two could not be regarded as homologous. The same may be said of the plasmodial stages of the Plasmodiophorales, for although their dimensions would be more nearly comparable, these also are never characterized by heavy cellulose walls. Furthermore, no one has yet reported a swimming stage, either gametic or vegetative, for any member of the Acrasieae and such are generally precedent to the formation of plasmodia in each of the other orders.

Here the matter must rest for the present, and definitive information regarding the true nature and ultimate significance of the macrocysts must await further research.

The writers are indebted to Miss Mildred M. Smith for her invaluable aid in the preparation of the illustrations used in this report.

Summary

1. Two encystment stages of cellular slime molds belonging to the genus *Dictyostelium* are described:

The first of these, termed *microcysts*, are unicellular and represent a transient resting stage in the vegetative phase of these simple slime molds. If returned to a favorable environment, microcysts germinated by excystment to re-initiate vegetative growth.

The second encystment stage, termed *macrocysts*, are multicellular and arise through a morphogenetic process possibly alternative to normal sorocarp formation. Myxamoebae aggregate to form typical but generally diminutive pseudoplasmodia which, instead of forming normal sorocarps, subdivide into rounded cell masses that become encased in relatively heavy cellulose walls. Concurrent with this de-

velopment, the myxamoebae that comprise the nascent macrocyst undergo limited differentiation and appear as polyhedral cells with highly refractive membranes. After a period of 10 to 14 days these so-called *endocytes* generally disappear whereupon the content of the macrocyst assumes an acellular, homogeneous appearance. With further aging the protoplasmic content shrinks away from the heavy cellulose wall and in this contracted stage retains its viability for protracted periods. Under favorable conditions of temperature and substrates, macrocysts of different ages germinate to release amoeboid cells which re-initiate the vegetative stage. The sequence of cytological changes underlying this behavior has not been elucidated, and this propagative function may or may not represent the full measure of their significance in the life-cycle of those slime molds which produce them.

2. The ability to produce microcysts is apparently inherent in all members of the Acrasieae, including the genus *Dictyostelium*. In contrast, the capacity to produce macrocysts is more restricted, having been observed only in occasional isolates of *D. mucoroides* and in many strains of *D. minutum*. Various environmental factors influence their production, and from one strain of *D. mucoroides* temperature-dependent "cyst-forming" and "spore-forming" clones have been isolated.

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PHYSIOLOGICAL OBSERVATIONS ON STARVATION AND DESICCATION OF THE SNAIL AUSTRALORBIS GLABRATUS

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It has been shown that planorbid snails, all of which are aquatic pulmonates, can withstand desiccation rather well both in nature and under laboratory conditions (Precht, 1939; Olivier, 1956a, 1956b; Olivier and Barbosa, 1955, 1956). The physiology of desiccating planorbids has, however, received scant attention. Magalhães Neto (1954) observed that five specimens of *Australorbis glabratus* showed a considerably decreased rate of oxygen consumption during desiccation at an unspecified relative humidity. Desiccating aquatic snails retract into their shells; they are unable to feed and hence come under conditions of starvation. Since starvation decreases the rate of oxygen consumption (von Brand, Nolan and Mann, 1948), and since the anatomical relationship of a retracted snail to the source of oxygen is quite different from that of an active one, the following questions arise: Is the reduction in oxygen consumption due mainly to starvation, to difficulties in securing sufficient oxygen, or to desiccation proper? These and related questions are discussed in the present paper.

Material and Methods

Laboratory-reared albino *Australorbis glabratus,* derived from a normally pigmented Venezuelan strain, were used in preference to pigmented specimens because the heart-beat could easily be seen through the shell. This was important, not only because a study of the heart rate under desiccation was interesting in itself, but also in order to establish whether a snail was alive or dead. The usual procedure of placing a desiccated snail in water to observe whether it resumes its normal activities could not be employed because in most of our experiments repeated measurements with the same specimens were required, or because a chemical determination had to be made on desiccated specimens. Most of the snails that appeared dead, as judged by cessation of the heart-beat, were tested further by placing them in water. Of about 200 such snails, only three revived, indicating that our death criterion was reasonably accurate.

All snails initially weighed between 180 and 350 mg. and had fed *ad libitum.* They were freed of excess moisture as described previously (Newton and von Brand, 1955) and weighed to the nearest mg. During starvation, snails (minimum of 28 per series) were kept individually in numbered beakers filled with dechlorinated tap water. They were shifted daily to fresh beakers during the first week of starvation and thereafter twice weekly. During desiccation, snails (minimum of

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33 per series) were put into individual dry beakers and these were kept at the same temperature (27 ± 1° C.) as the starvation series in desiccators over water or over saturated salt solutions giving a desired relative humidity. The solutions employed gave the following relative humidities, as determined by an Aminco electric hygrometer.

H_2O giving 95 to 97 per cent relative humidity, average 96 per cent
$ZnSO_4$ giving 83 to 87 per cent relative humidity, average 85 per cent
NaCl giving 72 to 76 per cent relative humidity, average 74 per cent
NaBr giving 57 per cent relative humidity, average 57 per cent
$CaCl_2$ giving 28 to 31 per cent relative humidity, average 30 per cent
LiCl giving 13 to 17 per cent relative humidity, average 15 per cent

When survival, weight, heart rate, and rate of oxygen consumption were determined, all snails alive on a given day were used. At the end of the determinations they were returned to the desiccators, or the water-containing beakers, respectively. These snails were used repeatedly until the last specimen died. When chemical determinations were done, only the specimens to be analyzed on a given day were used, while the others remained undisturbed until required for analysis.

The heart rate was determined by counting the heart-beats for one minute under a dissecting microscope.

The conventional Warburg technique was used for the oxygen consumption. The vessels contained 2 ml. of dechlorinated tap water in the starvation series and in the experiments designed to give the pre-desiccation rate. In the desiccation experiments the snails were put into the main compartment of the vessel without water. In these cases the side arms of all vessels, including thermobarometer, contained 0.3 ml. of the same salt solution that was present in the desiccators where the snails had been kept, thus maintaining approximately the same relative humidity. In all cases the temperature was 30° C.

Polysaccharides were determined according to von Brand's (1936) micro-modification of Pflüger's method. Total lipids were determined by heating the crushed snail with 30 per cent NaOH in a boiling water bath, acidifying the solution with 7 per cent H_2SO_4, extracting the solution three times with ether, washing the combined ether fractions with distilled water, evaporating the ether, and weighing the lipids on a microbalance after drying at 80° C. For lactic acid [2] and volatile acids the methods of Barker and Summerson (1941) and Bueding (1949) were used, respectively.

All measurements were done on numbered individual snails, with the exception of the volatile acid determinations, where two snails were used. All values are expressed on the basis of the initial, pre-experimental weight of the snails.

Results

1. Survival. Figure 1 shows the survival groups of starving snails at various relative humidities and in water. It is obvious that time of survival decreased with decreasing humidity. When the 50 per cent death times are plotted logarithmically against relative humidity (lower part of Fig. 1), no straight line

[2] We are indebted to Mr. C. Elwood Claggett for carrying out the lactic acid determinations.

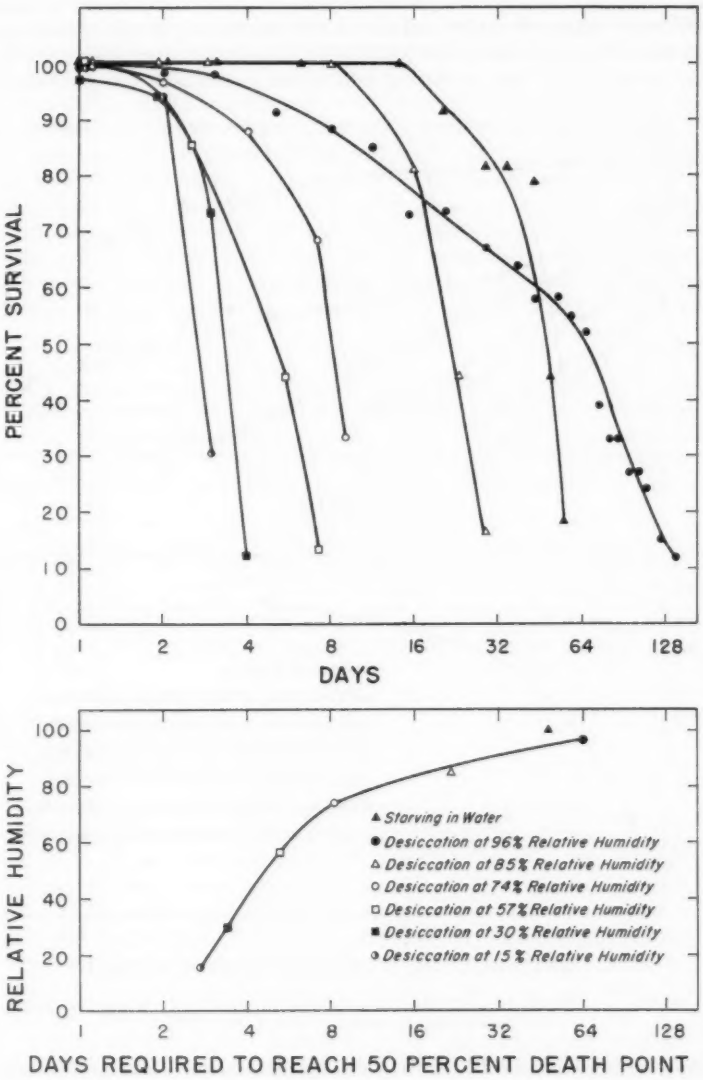

FIGURE 1. Survival of *Australorbis glabratus* starving in water or desiccating at various relative humidities. At the beginning each desiccating series consisted of 33 specimens, the water-starvation series of 28 specimens. The same groups of snails yielded the results shown in Figures 2, 3 and 4.

results. The shape of the curve indicates that no fixed relationship between time of survival and relative humidity exists, but that decreasing humidity leads progressively to an ever more accelerated death rate. Survival during starvation in water was only about half as long as during desiccation over water (96 per cent relative

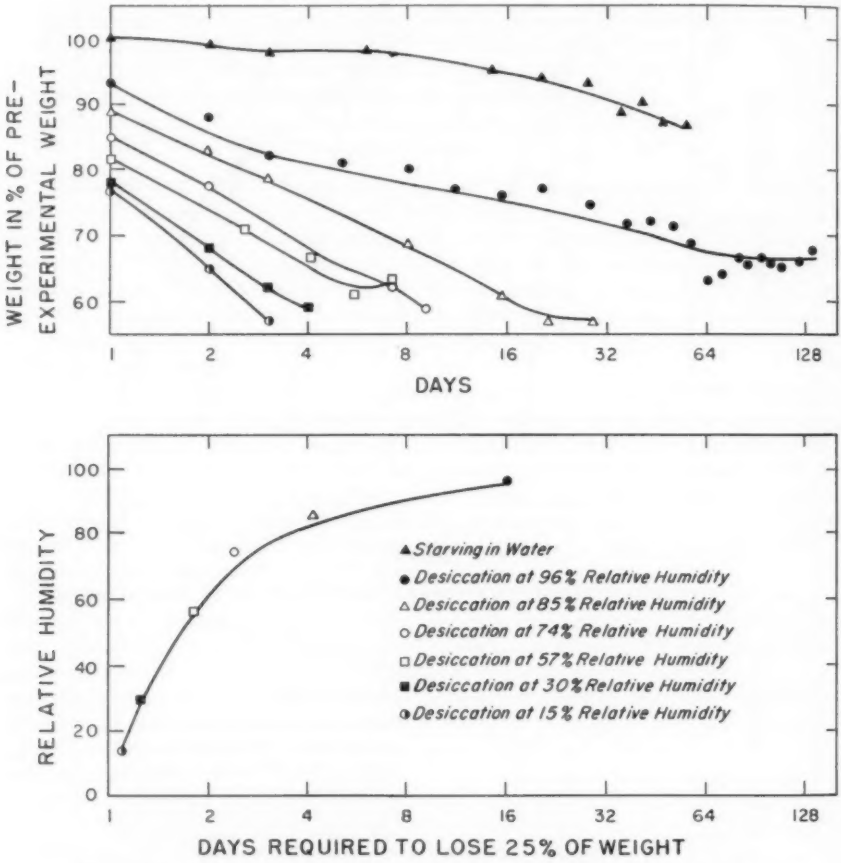

FIGURE 2. Weight relationships of *Australorbis glabratus* starving in water or desiccating at various relative humidities.

humidity). However, the 50 per cent death points of these two series were closer together. This is due to the fact that the first deaths due solely to starvation occurred later than those from the combined influences of starvation and desiccation.

2. Weight loss. Thirty fed control snails averaged 40.8 per cent dry substance. The shells of 30 other controls, after the soft parts had been removed according to Nolan and von Brand's (1954) procedure averaged 31.6 per cent of the total

weight. The dry weight of the soft tissues was thus 9.2 per cent of the total body weight. Since the shell contains practically no water, the total water found can be ascribed to the soft tissues. Their initial over-all hydration is then calculated as 87 per cent (Table III).

A total weight loss of 13 per cent was observed in snails starving to death in water. It was hence greater than the total dry weight of the soft tissues initially present and it must be concluded that it was due in part to a loss of water, perhaps corresponding to the hydration water of the metabolized organic material.

The weight loss of desiccating snails (Fig. 2) was much more pronounced than that of snails starving in water and was clearly dependent on the relative humidity. The lower part of Figure 2 indicates that the relationship between humidity and weight loss is very similar to that described above for survival. Snails desiccating and starving at 96 per cent relative humidity metabolize during 128 days approxi-

TABLE I

Desiccation of Australorbis glabratus at 85 per cent relative humidity. The figures are per cent of the pre-desiccation values; the figure following the ± sign is the standard error of the mean

Days of desiccation	Heart rate		Weight		Rate of O_2 consumption		Per cent survival
	A	B	A	B	A	B	
1	98±2.6	98±4.2	89±0.5	90±0.7	49±2.6	47±4.5	100
2	100±4.2	107±5.2	83±0.3	85±1.7	46±2.2	47±4.4	100
3	95±3.0	93±4.8	79±0.7	81±0.9	33±2.7	30±3.0	100
8	105±3.7	98±3.7	69±1.0	71±1.1	27±2.1	20±2.4	100
15	93±4.6	95±3.7	61±1.1	64±1.7	28±2.5	30±3.6	81
22	81±9.9	81±9.9	57±1.6	57±1.6	10±2.0	10±2.0	44
28	81±2.4		57±3.7		19±5.0		16

The initial number of snails was 33.
A = Values of all snails alive at specified day.
B = Values of all snails surviving on day 22.

mately 50 to 60 per cent of their organic material (corresponding to 4–5 per cent of the pre-experimental live body weight, see Discussion). Since in the humidity range of 15 to 85 per cent practically all snails had died by day 20, their loss of organic material must have been much smaller, and no appreciable error can be introduced if the entire weight loss is here ascribed to loss of water. At these humidities the weight had declined terminally to 60 per cent of the initial, corresponding to a loss of 40/59.2 = approximately 70 per cent of the initial water.

At 96 per cent humidity, on the other hand, survival was much longer and the weight at death was higher, amounting to 65 to 70 per cent of the initial weight. As mentioned above, about 4 to 5 per cent weight loss must in this case be ascribed to metabolized organic material. It is therefore clear that at this high humidity the last animals died before being desiccated to quite the same degree as in the other desiccation series. It is probable that in this case starvation was a contributing factor to death.

It should be realized that the figures summarized in Figure 2 (the following applies also to the data presented in Figs. 3 and 4) are averages for all snails

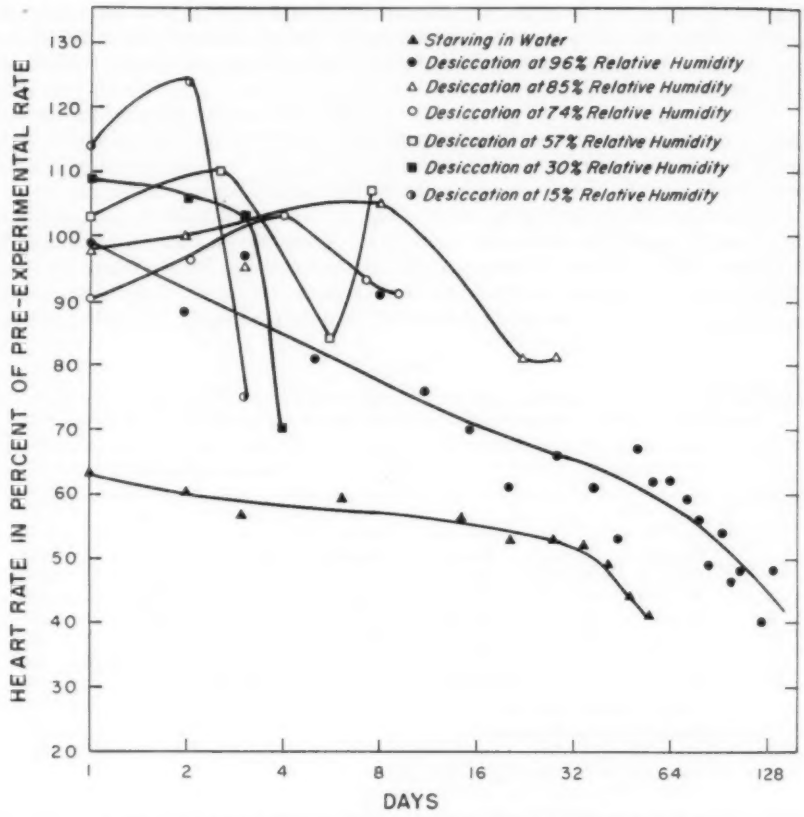

FIGURE 3. Heart rates of *Australorbis glabratus* starving in water or desiccating at various relative humidities.

alive at a specified day. They therefore represent the average changes occurring in a population. Some irregularities in the curves, especially noticeable towards the end of an experiment, are due to the summation of experimental errors and the slightly variable behavior of the individual snails. The curves do not change materially, however, if the data are restricted to snails which are still alive on a day nearing the end of a given experiment, provided the number is sufficient to give a valid average. This is illustrated for one of our series in Table I for weight and other criteria studied.

3. Heart rate. The heart rate of snails (Fig. 3) starving in water slowed precipitously to 63 per cent of the original rate during the first 24 hours of starvation. During the remainder of the starvation period the heart rate declined slowly further, the final value being about 40 per cent of the initial one. In snails desiccating at 96 per cent relative humidity there was no decline during the first 24 hours,

but thereafter the heart rate became progressively slower, reaching about the same end-point as in snails starving in water.

A different situation prevailed in snails desiccating at all lower humidities. Within the first few days, there was a period when the heart-beat increased in frequency above the pre-experimental value, this period being followed by one of more or less precipitous decline. While the heart-beat was generally full and regular in snails in water and in air at 96 per cent relative humidity, many irregularities were observed at lower humidities, such as partial contractions of the heart, or

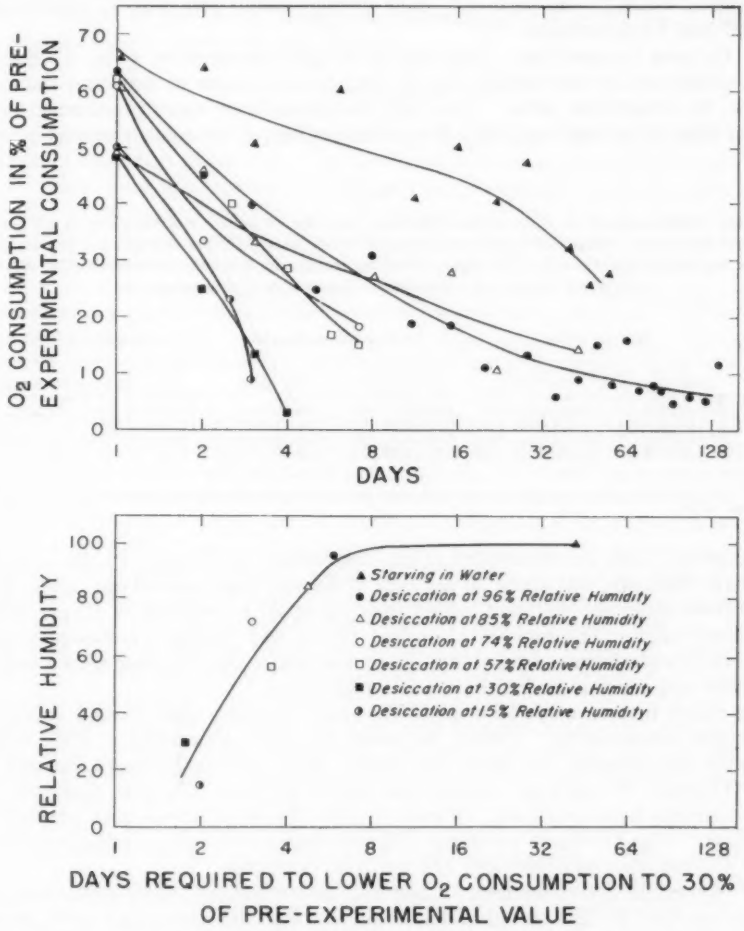

FIGURE 4. Oxygen consumption of *Australorbis glabratus* starving in water or desiccating at various relative humidities.

cessation of the pulsations for a few seconds followed by a period of very rapid contractions. It should be noted that at 57 per cent humidity the last value raises the curve (Fig. 3.) This artifact is due to abnormally high rates in all three surviving snails.

Heart rate apparently had no direct relation to survival under our conditions. For instance, in our series of 33 snails desiccating at 96 per cent relative humidity, 7 snails with initial heart rates of 33 to 37 beats per minute survived an average of 48 days. The other extreme was represented by 5 snails with initial rates of 50 to 63 beats/minute and an average survival of 80 days. After 128 days of desiccation, 5 snails still survived; their average initial rate was 42 beats/minute with 37 and 53 as extremes.

4. Oxygen consumption. The rate of oxygen consumption (Fig. 4) declined in all series more or less rapidly, but the decline was slower in the water-starvation than in the desiccation series. The daily variations were more pronounced in the former than in the latter, possibly due to the motility of the snails starving in water

TABLE II

Chemical determinations on Australorbis glabratus starving in water or desiccating at 96 per cent relative humidity. All values have been calculated on the basis of the pre-starvation or pre-desiccation live weight of the snails. The figure following the ± sign is the standard error of the mean, the figure in parenthesis indicates the number of determinations

Days	Per cent lipids		Per cent polysaccharides		μ Lactic acid	μ Volatile acid*
	Desiccation	Starvation	Desiccation	Starvation	Desiccation	Desiccation
0	0.76±0.023 (24)	0.65±0.036 (24)	1.03±0.10 (23)	1.29±0.16 (22)	140±27.8 (12)	53±28.1 (6)
10	0.58±0.019 (24)	0.43±0.026 (24)	0.77±0.07 (22)	1.04±0.18 (24)	117±28.1 (10)	11± 5.4 (6)
20	0.54±0.016 (20)	0.38±0.011 (19)	0.69±0.07 (24)	0.69±0.14 (21)	35± 7.9 (12)	18±12.8 (6)
30	0.52±0.019 (19)	0.36±0.011 (19)	0.56±0.08 (20)	0.59±0.12 (22)	0.0 (11)	23± 3.9 (3)

* The volatile acids are expressed as acetic acid, since this is the predominant volatile fatty acid (Mehlman and von Brand, 1951).

as contrasted with the immobility of the desiccating specimens. In the starvation series the final rate was about 30 per cent of the pre-experimental one, while in the desiccation series the endpoint varied from about 20 to well below 10 per cent of the initial value. The rapidity of decline (lower half of Fig. 4) showed a rough correlation with degree of humidity, but it was not so close as that shown between humidity and survival or humidity and weight loss.

A rough inverse correlation probably exists between survival and initial rate of oxygen consumption. Taking the series of snails desiccating at 96% relative humidity, as example, six snails had initial rates varying between 205 and 277 $mm.^3$ O_2/gm./ hr. with an average survival of 51 days. In four snails the rate varied initially between 82 and 108 $mm.^3$ O_2/gm./hr. and their average survival was 103 days. The five snails surviving 128 days desiccation had an initial rate of 143 $mm.^3$ O_2/gm./hr., with 106 and 171 $mm.^3$ as extremes.

5. Chemical determinations. Chemical determinations were performed only during the first 30 days on animals starving in water and desiccating at 96 per cent relative humidity since snails desiccating at lower humidities died too early. In view of the variability in storage of reserve substances, it was essential to limit

periods of exposure to experimental conditions to those tolerated by all or at least the great majority of specimens employed, since otherwise a possible differential death rate between snails with high and low initial reserves would make valid conclusions impossible.

The data summarized in Table II show that starving and desiccating snails use appreciable amounts of both polysaccharide and lipids, the consumption being more pronounced during the first 10 days than after prolonged exposure to experimental conditions. Snails starving in water used little more of these reserve substances than did the desiccating specimens. In desiccating snails the lactic acid initially present in the tissues disappeared completely within 30 days, while the volatile acid content decreased only slightly.

Discussion

The laboratory strain of *Australorbis glabratus* used in the present studies withstood desiccation fairly well. As was expected, the snails retracted into their shells. They were not capable of forming a true epiphragm which in many other species is an efficient mechanism for preventing excessive loss of water, nor did they produce complete mucus membranes across the shell aperture, an auxiliary mechanism frequently employed (Gebhardt-Dunkel, 1953). Partial mucus membranes were observed occasionally, but they did not seem to change the rate of evaporation materially.

Marked reduction in the rate of oxygen consumption with time was characteristic at all humidities studied and a loose inverse correlation with humidity existed. This reduction was not due solely, and in the series at low humidities not even primarily, to starvation. Snails starving in water maintained a higher rate of oxygen consumption than the desiccating specimens; they must therefore have used their reserve substances at a faster rate. It would then seem that the amount of reserve substances available to the desiccating animals would have sufficed to maintain an equal rate of oxygen consumption if starvation alone were involved. An altered anatomical relationship to the source of oxygen can also be eliminated as the cause of this reduction. If difficulties in securing oxygen played a significant role, a partial shift to anaerobiosis would have been expected. It should be noted in this connection that a partial shift to anaerobiosis can readily be induced in *Australorbis* by exposure to low concentrations of pentachlorophenol (Weinbach and Nolan, 1956) and that a considerable increase in lactic acid content has been reported from aestivating *Pila* (Meenakshi, 1956). In our desiccating specimens, on the contrary, the lactic acid present initially disappeared completely and the volatile acids diminished. There is little doubt that *Australorbis*, at least during desiccation at high humidity, maintained a purely aerobic metabolism despite the deep retraction into the shell.

The lung of a contracted snail is probably largely compressed and it is problematical whether it plays a large role in the gaseous exchanges. Diffusion through the tissues exposed to the air within the shell may have been sufficient. It should be kept in mind that conditions are quite different when a snail retracts into its shell in water. During desiccation the tissues are in direct contact with atmospheric air where the absolute amounts of oxygen are much higher than in water and where diffusion is incomparably more rapid than if the whorl is filled with water. It is

therefore most likely that the reduction in oxygen consumption was largely a consequence of desiccation proper, although in the longer-lasting series starvation may well have been a contributing factor.

That loss of water *per se* influences snails can be deduced also from our heartbeat observations. Starvation in water led to a reduction in rate, and a similar reduction, though slower to appear, was evident in snails desiccating at 96 per cent relative humidity. At lower humidities, on the contrary, the period of decline was preceded by one of increased rate and many irregularities in heart action were observed. While the final water loss was not very different, it was more gradual in the 96 per cent series and a difference in over-all tissue hydration was probably present even towards the end of the experiments (see below). On the whole, the impression was gained that at 96 per cent humidity the heart had an opportunity to adapt itself to changed conditions, while this did not occur during the shorter periods involved at lower humidities. It is probable that at the lower humidities increased concentration of organic and inorganic materials accumulating in the blood may have put a strain on the heart. It was not directly demonstrated because *Australorbis* is for technical reasons not suitable for such experiments, but Arvanitaki and Cardot (1932) had found previously a salt concentration of 0.080 N in *Helix pisana* collected immediately after a rain and 0.147 N seven days afterwards.[3]

In humidities of 85 per cent and below, the last snails died when they had lost about 70 per cent of their original water.[4] While this loss is very large,[5] the over-all tissue hydration does not decline to the same extent, because the remaining water hydrates the tissues of an animal whose weight has declined. The general relations between over-all tissue hydration and total water loss are shown in Figure 5. This figure is drawn on the assumption that the organic material remains unchanged, and is therefore valid only in cases of very rapid desiccation. In experiments of long duration, such as at 96 per cent relative humidity, a considerable percentage of tissue is lost. While no exact data could be secured, a final loss of 50 to 60 per cent appears possible (see below). If this loss is taken into account, the over-all tissue hydration was about the same at the beginning as at the end of the desiccation period (Table III). Even if this should be literally true (and no such claim is made), the physiological state of the desiccating snail probably differs from that of snails kept in water. For instance, any loss of water, whether accompanied by a loss of tissue or not, should result in an increased percentage of inorganic material

[3] It is probably unwarranted, however, to generalize: Pusswald (1948) reported that the blood of the slugs *Arion* and *Limax* lost 84.5 and 92.0 per cent of their initial water, respectively, when the water loss of the entire body was 60 per cent. In *Limax* the percentage of water content of the blood had at this point declined from 97.6 per cent to 77.3 per cent. In snails with external shells the water loss of the blood seems to be less pronounced. Gebhardt-Dunkel (1953) studied five species of terrestrial snails and found a decline in the water content of the blood from initial values ranging in the various species from 97.7 to 98.1 per cent to final values varying between 88.4 and 88.8 per cent shortly before death from desiccation.

[4] Actually, the water loss is probably slightly higher since the water resulting from the oxidation of food reserves has not been taken into account in this calculation.

[5] This resistance to loss of water is not unique. Roots (1956) states that the earthworms *Allolobophora chlorotica* and *Lumbricus terrestris* survive losses of body water of 75 and 70 per cent, respectively. Other invertebrates are more sensitive. According to Biancamaria (1955), the crayfish *Potamon edulis* dies after having lost 15 to 23 per cent of the original water. For older data on resistance to desiccation, see Hall (1922).

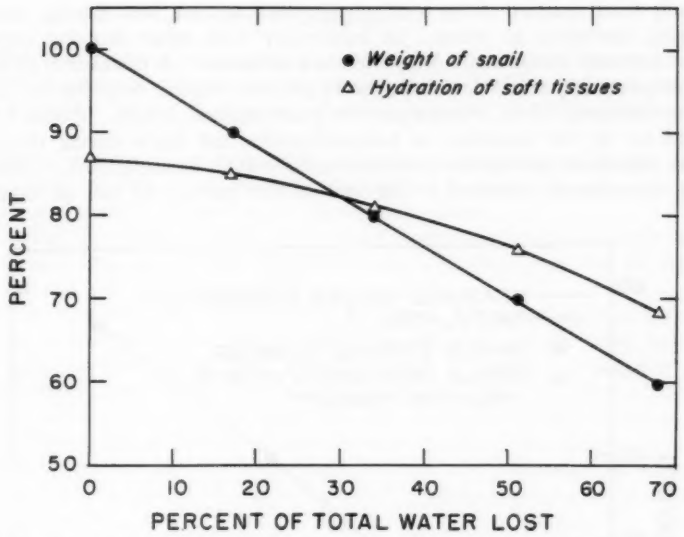

FIGURE 5. Theoretical relation between weight (water loss) and over-all hydration of soft tissues in desiccating *Australorbis glabratus*. Initial water content 87 per cent.

in the remaining tissues, unless the snail is capable of incorporating the excess into the shell, a point that was not studied. Computations from data presented by Buck and Keister (1949, Fig. 7) show that in flies, also, considerable water loss may occur without decrease in over-all tissue hydration. This suggests that the phenomenon may be widespread.

The endogenous foodstuffs during desiccation were studied only at 96 per cent relative humidity where a consumption of polysaccharides, lipids, lactic and volatile acids was found. Since there was no indication of a partial shift to anaerobiosis, total oxidation can be assumed and the oxygen required for it can be calculated. It is also possible to calculate approximately the total oxygen consumed during the desiccation period by graphic integration of the rates determined at the intervals shown in Figure 4. As Figure 6 indicates, the above substances account only for

TABLE III

Calculated over-all hydration of the soft tissues of Australorbis glabratus when 50 per cent of the soft tissues disappear during desiccation at 96 per cent relative humidity and the final total weight of the desiccated snail is 67 per cent of the pre-desiccation value, as was found

	Total weight, mg.	Shell weight, mg.	Weight of soft tissues, mg.	Weight of water, mg.	Per cent water in complex soft tissues+water
Pre-desiccation	100	31.6	9.2	59.2	87
Post-desiccation	67	31.6	4.6	30.8	87

a relatively small fraction of the total oxygen consumption, both during desiccation and during starvation in water. In conformity with other starving organisms, it may be assumed that proteins were the main substrate. A calculation of the total oxygen consumed by snails desiccating at 96 per cent relative humidity for 128 days gives approximately 56 ml. oxygen per one gram original weight. About 9 ml. are accounted for by the oxidation of polysaccharides and lipids during the first 30 days (the additional amount for lactic and volatile acids is negligible). Since these reserves were largely depleted at the end of this period, 47 ml. of oxygen can

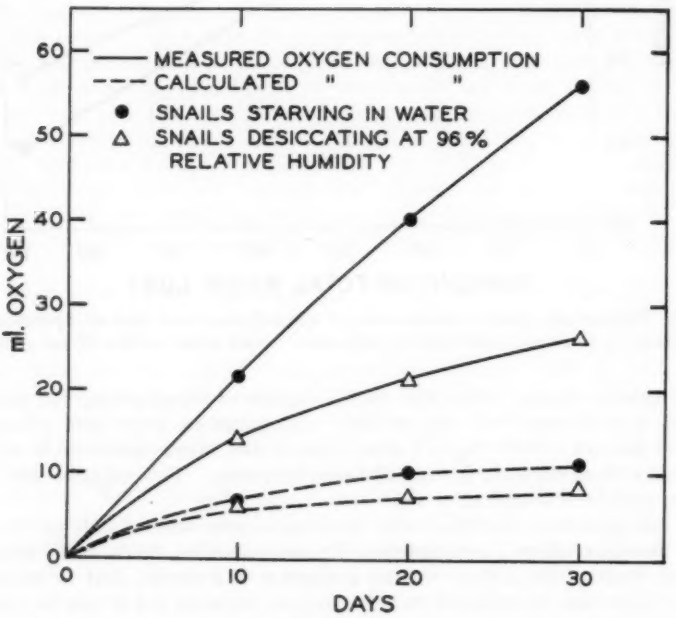

FIGURE 6. Comparison of oxygen consumption calculated from polysaccharide and lipid consumption, assuming total oxidation, and measured oxygen consumption (total oxygen consumption obtained by graphic evaluation of determinations done at specified intervals; see text). The values have been calculated for one gram pre-starvation or pre-desiccation weight.

tentatively be linked with protein consumption, permitting the oxidation of approximately 48 mg. of protein. Since a one-gram snail (initial weight) contains, on an average, 92 mg. of dried soft tissue, the calculated tissue loss would be roughly 50 to 60 per cent. This figure appears possible, since even higher organisms can lose more than 50 per cent of their weight during starvation, *e.g.*, a dog discussed by Pütter (1911) decreased in weight from 19.65 kg. to 9.17 kg., yet recovered upon feeding. Other examples are planarians, which decrease so markedly in size during starvation that their weight loss must be far larger than 50 per cent (Stoppenbrinck, 1905; Berninger, 1911).

Summary

1. Decreasing humidity leads to a progressively more rapid decline of survival time, body weight and rate of oxygen consumption. Snails starving in air of high humidity survive longer than snails starving in water, but their final weight is lower.

2. The heart rate of snails starving in water or desiccating at 96 per cent relative humidity decreases. At all lower humidities a transitory phase of increased heart rate and many irregularities in heart action occurs.

3. During starvation in water and during desiccation, polysaccharide and lipid stores become depleted. Lactic acid disappears completely from the tissues during desiccation and volatile acids diminish.

4. It is concluded that the decrease in oxygen consumption is largely due to desiccation proper but that at high humidity starvation is a contributing factor.

5. Snails desiccating at high humidity have a purely aerobic metabolism. The relationship between the oxygen required for oxidation of polysaccharides and lipids and the total oxygen consumed indicates that protein may be the main substrate during prolonged periods of starvation in water or of desiccation.

6. The percentage of total body water lost and the percentage of water in the tissues do not decrease at the same rate during desiccation, tissue hydration declining at a slower rate. If marked tissue losses occur during long periods of desiccation, the over-all tissue hydration may remain unchanged even if the total water loss is very pronounced.

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SIMILARITIES BETWEEN DAILY FLUCTUATIONS IN BACKGROUND RADIATION AND O_2–CONSUMPTION IN THE LIVING ORGANISM [1, 2]

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Recent studies on fluctuations in O_2-consumption and in spontaneous activity in conditions constant with respect to all factors known to influence organisms, have provided strong evidence that some external fluctuating physical factors are still exerting an influence on protoplasmic systems. The studies were made in conjunction with an analysis of temperature-independent, solar-day and lunar-day, cycles under constant conditions. Solar-day cycles have been known for a number of years to be widespread among organisms, and more recently it has become evident that lunar-day cycles also occur.

The evidence for an influence of an external factor has come from the recent rediscovery (see Stewart, 1898, for early literature) of correlations of organismic activities with barometric pressure and its changes (Brown, Freeland and Ralph, 1955; Brown, Webb, Bennett and Sandeen, 1955; Brown, Bennett, Webb and Ralph, 1956). These correlations have recently also been shown to occur in two lag-lead relationships. One is between the *rates* of barometric pressure change at certain specific times of day n, n-1, and n-2 as correlated with biological activity at an approximately corresponding time on day n. A second correlation is between the organismic activity at a particular time of day, expressed either in absolute terms or as deviation from the daily mean and the mean daily barometric pressure of the second day thereafter. That these correlations are in no manner responses to pressure changes themselves is clear not only from the lead-correlation of the organism on barometric pressure, but also from studies in which organisms were shielded from the normal external pressure fluctuations for as long as three consecutive months.

Recent work (Figge, 1947; Brown, Bennett and Ralph, 1955) has suggested that some form of cosmic radiation might be capable of influencing organisms. This view has been strengthened by the discovery of 27-day organismic fluctuations (Brown, Bennett, Webb and Ralph, 1956), a frequency recently reported to exist also in fluctuations in cosmic radiation (Simpson, 1954). As a consequence, the following studies were undertaken to investigate in some detail any possible relationships between general background radiation and organismic metabolism.

[1] These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and Northwestern University, NONR–122803.

[2] The authors wish to acknowledge their indebtedness to Professor H. T. Davis of the Department of Mathematics, Northwestern University, who gave freely very valuable advice during the course of the investigation and preparation of the material for publication.

Materials and Methods

The O_2-consumption of potatoes was recorded continuously from February 1 through May 31 at Evanston, Illinois by means of Brown (1954) recording respirometers modified in such a manner (Brown, 1957), that a constant pressure was maintained by hermetically sealing the respirometers and recording system in rigid copper containers, the barostats, in which the pressure was kept at a constant reduced level of 28.50 inches Hg. Five barostats, each with four respirometers jointly providing a single continuous record of the fluctuations in rate of O_2-consumption, were in essentially continuous operation during the four-month period.

A cylindrical core of potato with an eye was placed in each respirometer. These cores were about 2.2 cm. in diameter and 1½ cm. high. The first lot was prepared on January 31, and with the exception of a very few occasional single-potato replacements continued in the respirometers until May 1 when a completely new set of potatoes was substituted. These latter were followed through the month of May. Therefore, the first lot of potatoes remained in constant conditions including pressure for three months except for brief periods of 15–20 minutes once every two to six days when the O_2 reservoirs were being refilled and the CO_2-absorbent renewed. The second lot remained in constant conditions, with no replacements for one month.

Only complete, uninterrupted calendar-days of recordings were used in the analysis. Partial days of data (days a respirometer was set up) were discarded.

Background radiation was recorded continuously during the same four-month period by means of a 2×30-inch cosmic-ray counter with an appropriate scaler and data printer. This monitoring system, located in the same laboratory with the five barostat-respirometer ensembles, yielded a count rate of the order of 40,000/hour. A few days of data were missed about the middle of May.

Results

There were clear systematic fluctuations in O_2-consumption in the potatoes throughout the four-month period. These were most commonly ones appearing to possess a single major cycle a day. When 3×7-hour moving means of the average of all those two to five barostats for which recordings were complete on that day were calculated it was found that these daily fluctuations involved up to 28% increase, with a mean of 13.7% from lowest to highest values for the day for 30 sample days taken at random. In view of the leveling influence of the 3×7-hour sliding average, the actual range was undoubtedly substantially greater.

In Figure 1 (Nos. 1–5) are seen the mean forms of the daily fluctuation for the four-month period for each of the five barostats. It is quite evident that two general forms of mean daily fluctuation are apparent. Numbers 1, 3 and 4 showed a clear major cycle with a minimum in the early morning hours and a maximum in the late afternoon. Numbers 2 and 5 exhibited essentially a 180°-phase shift relative to the others. These five, independent, four-month samples, from lowest to highest values in the mean daily cycles are, respectively, 10%, 7.3%, 9.0%, 10%, and 4.8%. The average value of the five, 8.2%, is in remarkable agreement with the value, 8.0%, obtained for a two-month period in the spring of 1955 (Brown, 1957). All five mean cycles possess a minor peak about 6 P.M. and slight minima at 1–2 A.M. and 3 P.M., irrespective of the form of the major cycle. This fact is em-

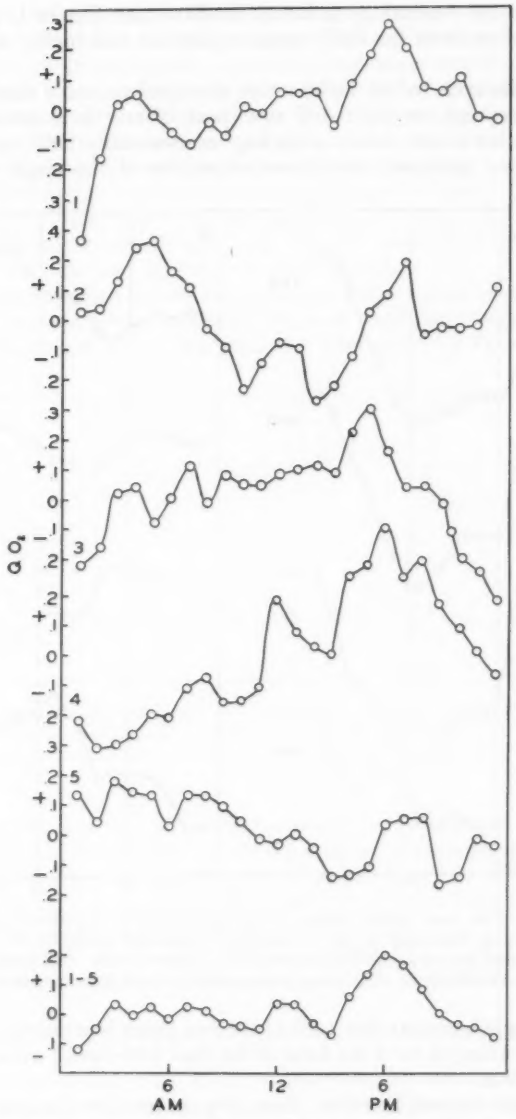

FIGURE 1. 1, 2, 3, 4, and 5 depict the mean daily cycles of fluctuation in O_2-consumption in potatoes, expressed as deviations from daily means, for each of the five independent respirometer-recording systems over the four-month period of study. 1–5 is the mean cycle for all the data. The actual percentage of the fluctuations is given in the text.

phasized in the mean 4-month cycle for all the barostats, Figure 1, (1–5) in which the 6 P.M. deviation from the daily mean is positive and highly significantly different from 0.

The mean daily cycle for all the barostats displayed no really significant evidence of a daily cycle except for the 5–6–7 P.M. peak clearly as a consequence of the algebraic summation of two forms of cycles, one essentially 180° out of phase with the other. It was apparent, also, from inspection of the single monthly mean

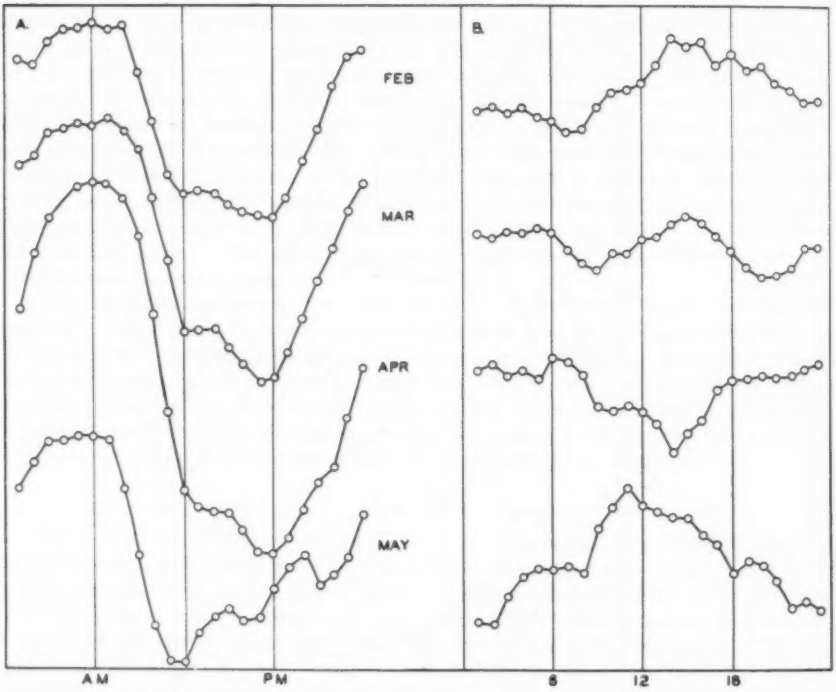

FIGURE 2. A. The mean daily cycles of general background radiation for each of four months. B. The mean lunar-day cycles of general background radiation for each of the four months. Lunar Zenith occurs at the 12th hour; Nadir, about 0 or 24. All these cycles are three-hour moving means of radiation. The mean percentages of the fluctuations are given in the text.

cycles for the single barostats that each of the five mean four-month cycles included monthly cycles tending to be of the form of the final four-month mean form, or 180° out of phase with it.

It seemed quite evident, therefore, that daily cycles of the potatoes were tending to exhibit one or the other of two forms, with, to the present, no suggestion that the occurrence of one type or the other is other than random.

An inspection of the fluctuations in radiation indicated there to be, in general, a clear mean daily cycle with a maximum about 6 A.M. and a minimum about 6

P.M. The mean daily cycles of three-hour moving means for each of the four months are seen in Figure 2 A. The mean cycles for February, March, and April are of strikingly similar form, with a gradually increasing amplitude (1.2%, 1.5%, and 2.3% increase from lowest to highest values). The maximum amplitude for a single day was about 10%. The cycle for May (5 days of data missing) showed an altered form though the amplitude, 1.3%, was of the same magnitude. Inspection of the daily data showed five days of the month (May 5, 15, 20, 23, 29) to have their cycles shifted about 180° relative to all the other days. The mean amplitude for the "typical" days was 2.2%, that for the five "shifted" days, 3.2%. Three days of the preceding month (April 1, 2, and 15) were also "shifted" days; no "shifted" days were present in February and March.

In view of the described presence of mean lunar-day cycles in numerous animals and plants, the mean lunar-day cycles of radiation were determined for each of the four months. These are seen in Figure 2 B. The cycles for February and March

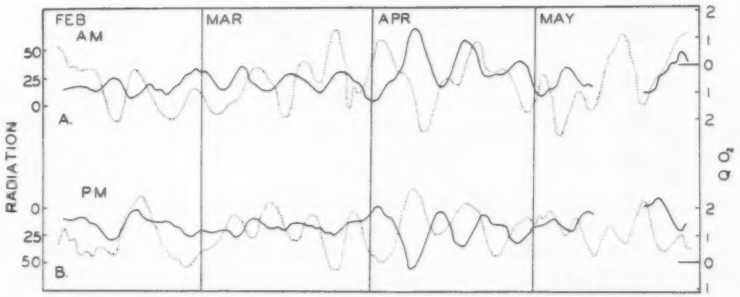

FIGURE 3. Five-day weighted (1:2:3:2:1) means of deviations of radiation intensity during the 2–6 A.M. and 2–6 P.M. periods from the daily means (solid lines) and corresponding weighted five-day means of O_2-consumption in potatoes for the 4–7 A.M. and 4–7 P.M. periods (dotted lines).

were rather similar, that of April shifted essentially 180° relative to the preceding two months. The cycle for May, with the highest amplitude of the four, again resembled those of February and March. There appeared to be a tendency for a maximum, or a minimum, to occur between the time of lunar Zenith and two to three hours afterwards, and for a maximum or a minimum to occur at the time of lunar Nadir. The amplitudes of the four average monthly cycles are, respectively, from minimum to maximum values, 0.57%, 0.37%, 0.60%, and 0.83%.

CORRELATION BETWEEN RADIATION AND O_2–CONSUMPTION

It is quite evident that even if the potato possesses mean daily cycles of O_2-consumption, its apparent tendency towards 180° phase-shifting would obscure much of this when large quantities of data were averaged. In view, however, of the relatively large-amplitude, daily fluctuations of the mean rates for all those potatoes recorded on a single day, an attempt was made to learn whether there might be a correlation between the amplitude of the day-by-day fluctuation in the background

radiation and the amplitude of the day-by-day fluctuation in O_2-consumption of the potatoes.

In Figure 3 A is shown a weighted (1:2:3:2:1) five-day moving mean of the deviations in intensity of radiation from its daily mean for the 2–6 A.M. period (all were positive values), and similar deviations from the daily means for the 2–6 P.M. period (all were negative values). Plotted on different ordinate scales are

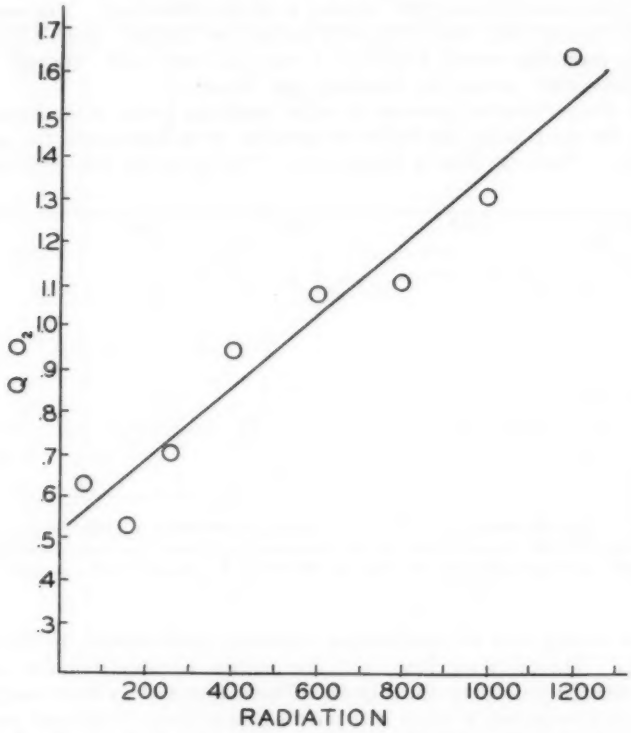

FIGURE 4. The relationship between the deviation in O_2-consumption at 4–7 A.M. and 4–7 P.M. from the daily mean of day n, expressed as deviations from monthly means, and the square of the deviation of radiation at 2–6 A.M. and 2–6 P.M. from its daily means for day n-1 expressed in the same terms ($P < 10^{-6}$).

superimposed correspondingly weighted five-day moving means of the deviation of O_2-consumption from the daily means for the 4–7 A.M. and 4–7 P.M. periods. It is evident from inspection that there is a highly suggestive similarity between the fluctuations in radiation and in O_2-consumption if one admits that the organismic cycles display alterations in sign of their correlation from time to time.

To quantify this similarity and to obtain at least an approximate measure of the significance of such an apparent similarity, a coefficient was determined for the

correlation between the deviations of radiation from its mean monthly deviations for each of the two times of day, on the one hand, and the deviations of O_2-consumption from their mean monthly deviations, for these times of day on the other. In this correlation, the signs of the deviations were ignored. A study of the regressional relationship indicated the relationship to be non-linear, and that the deviation in O_2-consumption was, instead, linearly related to the square of the deviation in radiation, and that the deviation in O_2-consumption on day n showed its correlations with day n-2, and especially n-1, of radiation with a rapid drop in coefficient to days n-3 and n. The coefficients and their errors for days n through n-3 were, respectively, 0.191 ± 0.066, 0.337 ± 0.062, 0.290 ± 0.064, and 0.135 ± 0.068.

The calculated regression for O_2-consumption on day n on the square of radiation for day n-1 is seen in Figure 4. The relationships between deviations in radiation and in O_2-consumption were calculated to be as follows:

Radiation	QO_2
0.5%	0.3%
1.0%	1.8%
1.5%	4.1%
2.0%	7.0%

The lag-lead relationship, radiation day n-1, was apparently simply the best compromise between radiation on day n-2 when using only the 4–7 A.M. value of O_2-consumption (0.41 ± 0.081), and radiation on day n using only the 4–7 P.M. value (0.369 ± 0.084).

An inspection of the form of the fluctuations in radiation and in O_2-consumption clearly suggested that in no other lag-lead relationship would correlations significantly different from zero be found over the four-month period. However, this was investigated more specifically. Correlations were found between radiation and O_2-consumption as follows: between the 4–7 A.M. deviation in O_2-consumption on day n from its mean 13-day deviation for this time of day, and the deviation of the 4–7 P.M. (day n-2) to 4–7 A.M. (day n-1) change in radiation from its mean monthly change for this period the coefficient was 0.352 ± 0.087. On the other hand, between the corresponding 4–7 P.M. deviation in O_2-consumption for day n and the corresponding 4–7 A.M. to 4–7 P.M. change in radiation of day n the coefficient was 0.382 ± 0.083. Eleven other lag-lead relationships for each of the times of day, sampling from day n-30 to day $n + 15$ for radiation failed to yield any correlations similarly highly significantly different from zero.

Discussion

The results which have just been described provide one additional kind of evidence in support of the conclusion reached by Brown, Freeland and Ralph (1955) and Brown, Webb, Bennett and Sandeen (1955), in a study of correlations between metabolism of various organisms and concurrent rates and directions of barometric pressure change, that the organism in "constant conditions" still is responding to fluctuations in some external physical factor or factors. This view was given further support by the studies of Brown, Bennett, Webb and Ralph (1956) on the quahog. In more recent studies (Brown, 1957) employing barostats as in the current study, correlations with barometric pressure were found

for O_2-consumption in potatoes over about a 1½-month period of study. In this latter investigation, unlike in earlier ones, there was a strong indication that the fluctuations in O_2-consumption on any given day tended to display either one of two patterns, one tending to be 180° out of phase with the other for either the whole or part of the day.

In the last-mentioned study there was a correlation of the deviations (ignoring sign) from the daily mean of the O_2-consumption of the potato at 4–7 A.M. on day n with the rate and direction of barometric pressure change from 2 to 6 A.M. centered on day n-2. In another kind of analysis of the data, there was found to be for the potato, a correlation between the 4–7 P.M. deviation from the daily mean of O_2-consumption and the mean barometric pressure on day $n + 2$ (Brown, Webb and Macey, 1957). Despite these correlations, the directly effective factor, in view of the use of barostats, could not have been pressure *per se*.

It is interesting that in this current four-month study there was also clearly reproduced a positive correlation between the deviation of the 4–7 P.M. value of O_2-consumption on day n from its daily mean and the mean barometric pressure of day $n + 2$ (Brown, Webb and Macey, 1957). It will be recalled that the 4–7 P.M. period was the only period of the day possessing a non-inverting cyclic component, and the correlation with barometric pressure correspondingly remained positive throughout the whole four-month period. In the spring of 1954, when a comparable and relatively striking negative correlation (-0.65) was found using the potato (not in barostats), it was similarly only the 4–7 P.M. period of the day which possessed this property.

Another notable observation made in the current studies is that the correlation with the deviations in radiation were maximum for days n to n-2(n-2, for the 4–7 A.M. O_2-consumption) for radiation. This is essentially the same lead-lag relationship found for the potato in the spring of 1955, a year earlier, with rates of barometric pressure change for comparable periods of the day, and also which obtained during the summer of 1955 for the sea weed, Fucus (unpublished results).

It seems reasonable to postulate that the living organism is displaying a mean one- to two-day lag response to some external factor correlated both with fluctuations in barometric pressure and with daily cycles in background radiation, and that the effective external fluctuations are in some manner correlated with the mean daily barometric pressure on the third to fourth day thereafter. From the standpoint of a possible significance of these external factors for the maintenance of the precision of the many known regular daily cycles observed to persist for long periods under constant conditions, it must be admitted that the organism can exhibit a metabolic response to some external factor which has clear mean daily cycles, even though with a randomly fluctuating amplitude. It has been postulated earlier (Brown, 1957) that organisms, through an endogenous capacity to oscillate, are able to maintain in many instances an endogenous cycle of the same frequency as the external ones.

The mean lunar-day cycles of radiation are of special interest relative to lunar-day mean cycles of biological activities which have been described (*e.g.*, Brown, Freeland and Ralph, 1955; Brown, Webb, Bennett and Sandeen, 1955; Brown, Shriner and Ralph, 1956). The principal maximum (or minimum) in both the mean lunar-day cycles of radiation and of most of those biological activities so far described appear to occur at the times of lunar Zenith or shortly afterwards. The

results encourage a more detailed study of lunar-day relationships of radiation and activity comparable to the current one for solar-day relationships. Such a study is in progress. Also, very suggestive in this regard is the observation that the ratio of amplitude of the mean solar-day cycles to the lunar-day ones of radiation, is of the same order as the ratio of the amplitude of the solar-to-lunar-day cycles of most of the reported mean cycles for organisms, namely 2 or 3 to 1.

Summary

1. O_2-consumption of potatoes was recorded continuously through the four-month period, February-May, 1956.

2. There were daily fluctuations in rate which, even using 3×7-hour moving means, displayed a mean amplitude of about 14%.

3. Five independent respirometer-recording systems yielded mean daily cycles for the four-month period ranging in amplitude from 4.8 to 10.0% with a mean of 8.2%.

4. The mean cycles were of two forms, one essentially 180° out of phase with the other.

5. Solar-day and lunar-day mean fluctuations in background radiation were also determined for the period of the investigation.

6. A small but very highly significant correlation existed between the fluctuations in amplitude of the daily cycles in radiation on day n-1 and amplitude of the daily fluctuations in O_2-consumption in the potato on day n.

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LAG–LEAD CORRELATIONS OF BAROMETRIC PRESSURE AND BIOLOGICAL ACTIVITY[1, 2]

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It has been known for many years that numerous kinds of organisms representing most of the major divisions of living things exhibit under constant conditions overt cyclic fluctuations of solar-day frequency of one or more of their processes. Less well known is the fact that at least a few organisms which live in the intertidal regions of the oceans have similarly clear cycles of tidal frequency. These rhythms appear to be expressions of a biological-clock system upon which the organism normally relies importantly in the adaptive regulation of its physiological behavior in its rhythmic external environment. In the past few years it has been firmly established that the frequencies of these cycles are independent of temperature over a wide temperature range, a characteristic which is, of course, an essential one if these cycles were to have practical adaptive value in the normal environment with its substantial fluctuations in temperature from hour to hour and day to day. Particularly within the past two years it has become more and more evident that all animals and plants have average solar-day and lunar-day fluctuations and that in the maintenance of these cycles, the organisms are receiving stimuli of some character from the fluctuating external physical environment even under conditions generally considered to be constant.

Evidence for an influence of a fluctuating external factor affecting the organisms even under "constant conditions" has come from both 1) highly significant correlations between hourly rates of metabolism in several organisms and concurrent hourly barometric pressure changes, and 2) remarkable similarities in the forms of day-to-day changes in mean rates of metabolism for the whole day, or specific parts of a day, and the forms of the day-to-day large climatic changes in the mean daily barometric pressures (approximated by the pressure for any arbitrarily selected, restricted, time of day). The day-to-day drifting of mean barometric pressures in a temperate-zone area appears superficially to be random, with every given period a month or so long, exhibiting its own specific pattern. Despite this, the forms of the day-by-day fluctuations in rates of metabolism or certain other biological phenomena of a number of species of organisms have appeared to be rather similar, either in a direct relationship or an inverse one, to the concurrent fluctuations in mean daily pressures. This has been found true to such an extent

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as to seem more than fortuitous, though there is no generally acceptable test for significance of correlations of such time series.

For many years relationship between barometric pressure and human physiology has been believed to exist. As a result of the prevalence of such a view during the first half of the last century Vivenot (1860) performed what he considered a crucial experiment to test this view. He constructed an air-tight chamber within which an experimenter could study pulse and respiration rates in subjects while the pressure within the chamber was experimentally altered. With this he found no evidence that small pressure changes had any influence upon the individual. A few years later, Lombard (1887) described striking similarities between fluctuations in the strength of the normal knee-jerk and concurrent fluctuations in external barometric pressure and temperature. The correlation was positive with pressure and negative with temperature. Later, the same investigator (Lombard, 1892) in more extensive studies reported a correlation between the rate of fatigue for voluntary contraction of the flexor muscle of the second finger and barometric pressure. With rising pressure there was increased capacity for work and with falling, decreased. The biological phenomenon retained its correlation with both the regular daily tidal and the irregular climatic pressure changes. Furthermore, pressure changes experimentally obtained by ascending and descending a mountain yielded quite comparable correlations.

Some experiments with spontaneous activity of other mammals yielded comparable apparent relationships to barometric pressure changes. Hodge (1897), studying the spontaneous running of two dogs through the application of special pedometers to their collars, found a correlation between the mean daily activities of the two dogs with respect to one another and to the concurrent mean daily barometric pressure. With the latter the correlation was positive over the two-month period of study. The correlation with pressure was extensively confirmed by Stewart (1898) employing rats and certain other mammals, in activity recorders. Gray rats exhibited a negative correlation with pressure over the 70-day period of his study. White rats, for a 30-day period included within the previous study period, showed a positive correlation with pressure. Stewart postulated, on the evidence at hand, that wild mammals displayed a negative, and domesticated animals a positive, correlation with pressure.

A widespread occurrence of a correlation between barometric pressure changes and biological activities was found by Brown, Freeland and Ralph (1955) and Brown, Webb, Bennett and Sandeen (1955) who were investigating fluctuations in O_2-consumption in potatoes, carrots, seaweed, fiddler crabs, and salamanders. All of these organisms showed correlations highly significantly different from zero between the hourly rates of O_2-consumption and the concurrent rate and direction of barometric pressure change. Later, Brown, Bennett, Webb and Ralph (1956) found correlations between the spontaneous opening of oysters and quahogs, and barometric pressure changes. In these recent studies it was strongly suggested by inspection of the data, however, that in paralleling the patterns of fluctuations, the organisms were actually leading the barometric pressure changes by more than a day.

One purpose of this report is to describe results from a simple statistical analysis of all the results on this score obtained in our laboratories between March 1, 1954 and June 9, 1955. These point conclusively to an ability of a wide variety of living

TABLE I

Organism and place	Dates (inclusive)	Correlation times Organism/Bar. pressure	r
Fucus[1] Woods, Hole, Mass.	July 28–Aug. 2 } 1954 Aug. 21–29 }	day n / $n+2$ (5–7 A.M.) (5–7 A.M.)	-0.476 ± 0.200
	Aug. 3–20, 1954	day n / $n+2$ (5–7 A.M.) (5–7 A.M.)	$+0.507\pm0.176$
Ostrea (10° C.)[2] Evanston, Ill.	Mar. 1–April 13, 1954	day n / $n+1$ (av. daily) (av. daily)	$+0.411\pm0.125$
Ostrea[3]	June 18–July 28, 1954	day n / $n+2$ (av. daily) (av. daily)	$+0.383\pm0.139$
	July 29–Aug. 27, 1954		-0.658 ± 0.104*
Rattus[6]	Nov 16–Dec. 3 } 1954 Feb. 2–Mar. 13 } 1955	day n / $n+7$ (noon) (noon)	$+0.598\pm0.068$*
	Dec. 5–Feb. 1, 1954–55	day n / $n+7$ (noon) (noon)	-0.362 ± 0.113
Solanum[1] Evanston, Ill.	May 12–June 9, 1954	day n / $n+1$	-0.650 ± 0.110*
Solanum[6] Evanston, Ill. (5 groups)	April 1–June 8, 1955	day n / $n+2$ (5–7 P.M.) (5–7 P.M.)	$+0.266\pm0.071$
Triturus[4] Evanston, Ill.	May 12–June 9, 1954	day n / $n+2$ (5–7 P.M.) (5–7 P.M.)	-0.842 ± 0.059*
Uca pugilator[4] Woods Hole, Mass.	June 20–July 20, 1954	day n / $n+2$ (5–7 A.M.) (5–7 A.M.)	-0.472 ± 0.144
	July 21–Aug. 27, 1954	day n / $n+2$ (5–7 A.M.) (5–7 A.M.)	$+0.595\pm0.104$*
Uca pugnax[4] Woods Hole, Mass.	June 18–Aug. 29, 1954	day n / $n+2$ (5–7 P.M.) (5–7 P.M.)	-0.423 ± 0.100
Venus[5] Woods Hole, Mass.	June 18–Aug. 29, 1954	day n / $n+2$ (av. daily) (5–7 A.M.)	-0.446 ± 0.096

[1] Brown, Freeland and Ralph (1955).
[2] Brown (1954).
[3] Brown, Bennett, Webb and Ralph (1956).
[4] Brown, Webb, Bennett and Sandeen (1955).
[5] Brown, Shriner and Ralph (1956).
[6] Brown (1957).

* Conventionally determined standard errors for such large values of r do not provide true measures of probabilities.

things, ranging from lower to higher plants and from lower to higher animals, to show a lead correlation with barometric pressure changes by one to seven days (usually two). The only organism studied in our laboratory during this period which was not included in this report is the carrot, which was omitted, not because of any lack of similar type of correlation, but rather because there were some known injury effects on the O_2-consumption during part of the single-month period of study.

The organisms, the times of study, and the coefficients of correlation obtained in 703 organism-days are given in Table I. These are all correlations of three-day moving means except for the potato in 1955, in which daily mean values of O_2-consumption were correlated with three-day sliding averages of barometric pressure. Indicated in footnotes are the references to the publication of the results obtained with these species. In these publications it had not occurred to the authors to make this type of analysis, and furthermore, due to the extraordinary nature of the conclusions, it is to be doubted that a single demonstration of the phenomenon of a lead correlation, even though shown to be statistically significant (assuming random fluctuation in the organism), would have been credited by most physiologists.

All of the results were obtained in conditions of constant, continuous low illumination of the order of 1 ft. c. or less, except with the white rat, *Rattus*, for which two periods of continuous darkness, totalling 43 days, were included in the data. The conditions of the experiments with the brown alga, *Fucus*, two species of fiddler crabs, *Uca pugnax* and *Uca pugilator*, the salamander, *Triturus* and the potato, *Solanum* included also very precisely regulated constant temperature, and, in addition, for *Solanum* in 1955, constant pressure through the use of a barostat.

The biological process studied was the rate of O_2-consumption in *Fucus*, *Uca pugnax* and *Uca pugilator*, *Triturus* and *Solanum*. It was the average daily minutes open per hour for both the oyster, *Ostrea* and the quahog, *Venus;* it was the total distance spontaneously run per day in the case of *Rattus*.

To determine when the correlation would be highest, whether with the same day (day n) of barometric pressure, the following day (day $n + 1$), the second day after (day $n + 2$), or some earlier or later one, tracings of the fluctuations of the physiological process and of barometric pressure were superimposed and inspected in various temporal relationships. In the vast majority of cases, there was clearly only one relationship which promised high correlation with an obviously rapid drop towards no correlation with either greater or less displacement and no other relationship with nearly as high a correlation could be seen by such inspection over the rest of the period. In two cases, *Triturus* and *Solanum*, it was not evident from inspection, whether the correlation would be best with day $n + 1$, $n + 2$, or day $n + 3$. In these cases, coefficients were determined for all three times and the highest one was selected; it was obvious, however, there was no other relationship with these species in which the correlation would be as high or significant.

The change in sign of the correlation from time to time seems clearly to be a biological contribution, probably to be compared superficially with the well-known changes of sign frequently observed in animal orientation. The sign change appears to occur abruptly and cleanly; the cause of the change is still unknown. It is known, however, that in all those cases examined in which the sign of correlation

changed, there was concurrently a transformation of the form of the mean daily and lunar-day cycles to essentially their mirror images.

For all the periods the correlations ranged in size from 0.266 to 0.842. All were significantly different from zero by ordinary tests for correlations. Both the − 0.362 for *Rattus* and the 0.383 for *Ostrea* appeared to include one or two very brief periods of change of sign in the relationship. The potatoes in 1955, judging by periodic inversions of their solar-day cycles, appeared to be changing the sign of their correlation from time to time, and probably hence the lowest, though quite real, correlation.

The great majority of the correlations centered on day $n + 2$ of barometric pressure. *Solanum* in 1954 and *Ostrea* at 10° C., centered on day $n + 1$. The potato in 1955 gave almost the same correlation for $n + 2$ and $n + 3$. The most surprising result, on the basis of hypotheses available to account for this phenomenon, was that *Rattus* showed by far its highest correlation with day $n + 7$. The coefficient would have risen from 0.598 ± 0.088 to 0.668 ± 0.07 by the justifiable statistical procedure of eliminating from consideration the transitional values, those of Dec. 1–3 and Feb. 2, which were seen clearly to contribute naturally to neither series.

Since there appeared to be no test for the significance of correlations between two time series that would be acceptable to all statisticians, further experiments were performed to attempt to demonstrate the reproducibility of the phenomenon. The first of these involved a four-month study of potatoes in Evanston, Illinois. Small cores of potatoes bearing eyes were obtained by means of a large cork-borer, and one was placed in each of 20 respirometer vessels (Brown, 1954). Four respirometer vessels upon a single recording system were sealed in each of five barostats on Feb. 1, 1956. During a three-month period, the respirometers were opened for about 15 minutes once every two to six days, to refill the O_2-reservoirs and replace the CO_2-absorbent. Very rarely a potato was replaced with a new one during this period. On the first of May, a completely new lot of potatoes replaced the old, and the observations were continued through May 31. During this study the potatoes were maintained in constant conditions of temperature (19.5° C.), of light (< 0.5 ft. c.), and of all other factors known to influence organisms. The respirometers were kept under a constant reduced pressure of 28.50 inches Hg. Approximately 56,000 organism-hours of O_2-consumption were obtained.

Inspection of three-day moving means of the mean daily barometric pressure for the four-month period, and comparison of weighted (1:2:3:2:1) five-day moving means of the 4–7 P.M. deviation in rate of O_2-consumption from the daily mean, gave clear suggestion that just as with the potatoes in the 1954 and the 1955 experiments, there was a lag correlation of barometric pressure on O_2-consumption by about two days. A scatterplot of the relationship between the barometric pressure of day $n + 2$ and O_2-consumption on day n is seen in Figure 1. This yielded a correlation coefficient of 0.339 ± 0.0835, a value highly significantly different from zero.

Figure 1, B, illustrates the various values of r obtained in various lag-lead relationships between the two phenomena, *i.e.*, for O_2-consumption of day n correlated with barometric pressure in various temporal relations from day $n - 30$ to day $n + 15$, a 45-day span. In this instance not only was a correlation centered

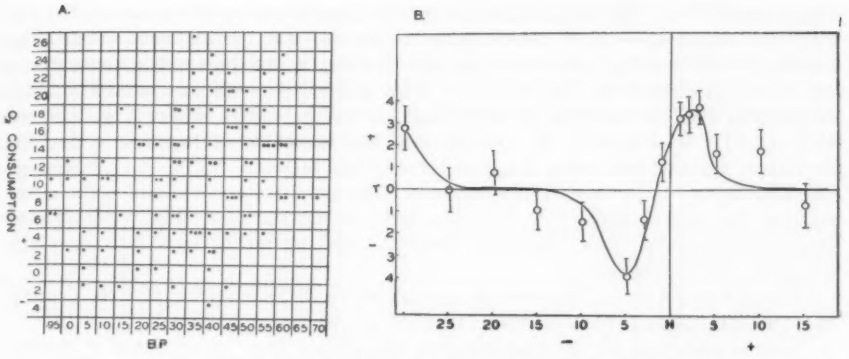

FIGURE 1. A. Scatterplot of the relation between the mean barometric pressure on day $n+2$ and the 5–6–7 P.M. deviation from the daily mean of O_2-consumption of the potato on day n. B. Coefficients of correlation (ordinate) between the 5–6–7 P.M. deviation in O_2-consumption from the daily mean on day n and the mean barometric pressure on various days from $n-30$ to $n+15$ (abscissa).

on day $n+2$, but a correlation was also found with barometric pressure, day $n-5$.

A second attempt was made in 1956 to confirm an organismic lead-correlation of metabolism on barometric pressure. This one was performed in Woods Hole, Mass., between June 16 and August 1. These observations were made upon fiddler crabs, whose O_2-consumption was measured under constant conditions including pressure in the same type of apparatus as that used for the potatoes. In this study of the fiddler crab, the temperature was similarly very constant but at a higher

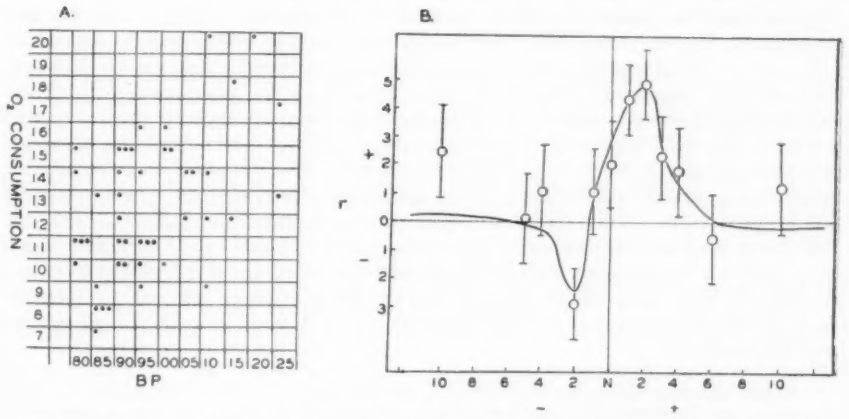

FIGURE 2. A. Scatterplot of the relation between the mean barometric pressure on day $n+2$ and the 5–6–7 P.M. value of O_2-consumption of the fiddler crab on day n. B. Coefficients of correlation (ordinate) between the 5–6–7 P.M. value of O_2-consumption from the daily mean on day n and the mean barometric pressure on various days from $n-10$ to $n+10$ (abscissa).

value, near 24° C. In this analysis, as for the experiments performed in 1954 and 1955, the actual rates of O_2-consumption at the 4–7 P.M. period were used. The scatterplot relationship between respiration on day n and barometric pressure on day $n + 2$ is shown in Figure 2, A. This yielded a coefficient of 0.49 ± 0.12, remarkably close, coincidentally, to the mean of all the values obtained in 1954 and 1955 (0.5). In Figure 2, B, are depicted the values for r together with their deviations for this and other lag-lead correlations between day n of O_2-consumption and day $n - 10$ to $n + 10$ for pressure. The only real correlations in the series are for days $n + 1$ and $n + 2$, with the latter being the more highly significant. In these correlations with the crabs three-day moving means were used for both pressure and metabolism.

These two series of experiments performed in 1956 would appear to confirm in a striking manner the conclusions reached in the earlier studies.[3]

The explanation of the phenomenon considered here seems from a general standpoint to be quite evident. Since the organisms cannot be determining the barometric pressure changes which are to occur, the organisms must be responding to some physical factors and their fluctuations which themselves exhibit a lead-correlation on barometric pressure. The potatoes in 1955 and the potatoes and crabs in 1956 remained in each case in barostats at 28.50 inches Hg from one to three months, the barostats opened only for about 15 minutes once every two to six days, and hence, the lead correlation cannot be due to any special responses to current rates of change in pressure itself. Since organisms have been shown to possess fluctuations in metabolic rates correlated with the rates and directions of barometric pressure change and especially since 27-day cycles have been found, it appears suggestive that the organism may be able to respond directly to fluctuations in the intensity of some high energy radiation or of some other physical factor with radiation-correlated fluctuation.

It is futile at the present time to do much speculating as to the external forces involved and the manners in which fluctuations in them may interact with the now established solar-day and lunar-day clocks and average cycles within organisms. This is now being investigated. It may be shown eventually that the phenomenon described in this report depends in some manner on the possession by both the organisms and the atmosphere of a solar-day and lunar-day cyclicity, and the interaction of these with some less orderly cosmic factor to which both the organism and atmosphere can react in an oscillatory fashion. But irrespective of the detailed mechanism, correlations of the order of magnitude described here (nearly 0.50 as the average degree of correlation for eight species of animals and plants over about 850 days), and tending very strongly to be centered on day $n + 2$, especially since they cannot be correlations with an actual causative force, are to be viewed as extraordinary. They force one to conclude that the living organism is clearly responsive in an orderly way to forces not hitherto seriously considered by biologists to possess any influence.

[3] Since the manuscript was completed, a further study of the potato during October, November and December, 1956 also yielded a lead correlation in which the highest correlation (-0.400 ± 0.089) was similarly found with the mean barometric pressure of day $n + 2$, rapidly falling on days $n + 1$ and $n + 3$ to -0.307 ± 0.094 and -0.328 ± 0.093, respectively, and on days n and $n + 4$ to a value not significantly different from zero. From inspection there was no other lag or lead relationship in which a significant correlation existed.

For the biologist who is attempting to account for the remarkable capacity of organisms to measure off with great precision under so-called "constant conditions," temperature-independent cycles of the frequencies of natural external cosmic events, it becomes highly important to know whether the conditions are truly constant for the organism, and if not, what is the actual character of the fluctuations in the effective external factor or factors.

Summary

1. Eight species of living things, ranging from lower to higher plants and lower to higher animals, in studies over a three-year period and including approximately 850 species-days have exhibited without exception a statistically significant lead-correlation on barometric pressure with an over-all mean coefficient of about 0.5.

2. The correlation involved sometimes only the 5–6–7 A.M., sometimes only the 5–6–7 P.M., and other times the mean daily rates of O_2-consumption.

3. The sign of the correlation was sometimes positive and other times negative. Sign changes, when they occurred during a single period of study were abrupt, and correlated with a 180°-shift in the phase relationships of the concurrent mean solar-day cycles.

4. In twelve periods of study, ranging from one to four months each, the correlation in nine cases centered on day $n + 2$ of barometric pressure. In two cases it centered on day $n + 1$ and in one, on day $n + 7$.

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THE LUMINESCENCE OF THE MILLIPEDE, LUMINODESMUS SEQUOIAE [1]

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The millipede *Luminodesmus sequoiae* was first described by Loomis and Davenport (1951). Following this, Davenport, Wootton and Cushing (1952) described the biology of the animal and the general nature of its luminescence. They found that light emission originates from cells in the deeper layers of the integument. The present paper describes a more detailed study of its luminescence.

The bioluminescent reaction in four different organisms (*Cypridina*, fireflies, bacteria and *Gonyaulax*) has been studied in recent years (Tsuji, Chase and Harvey, 1955; McElroy and Hastings, 1955; McElroy and Green, 1956; Hastings and McElroy, 1955; Strehler, 1955; Hastings and Sweeney, 1957). The common feature is that the reaction involves an enzymatic oxidation with molecular oxygen. Although it was demonstrated by McElroy that adenosine triphosphate is an absolute requirement for firefly luminescence, its possible role as an energy source in the reaction has not been clarified. In bioluminescent reactions in general it is assumed that the energy must be derived from the oxidation of a substrate, which is usually termed luciferin (*e.g., Cypridina* luciferin, firefly luciferin, etc.). None of the products of luminescent reactions have been definitely identified and the reactants as well as the enzymes are different in all cases studied. Such studies are of interest for the general problem of how the living cell transforms chemical energy into other forms of energy.

Materials and Methods

About 1000 animals were collected by a party of five on the nights of May 10 and 11, 1956, in the vicinity of Camp Nelson, Tulare County, California. The animals were abundant and readily visible to the dark-adapted eye by their own light on the surface of the ground in the forest. The animals were brought back to the laboratory and stored in glass containers with ample humus. Light intensity was measured with apparatus previously described (Hastings, McElroy and Coulombre, 1953), using a photomultiplier tube and automatic graphic recording.

Results

1. In vivo luminescence

Luminescence in *Luminodesmus* is continuous but fluctuating. The light intensity (recorded from single animals over long periods up to 24 hours) fluctuates

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by 20 to 40 per cent (or occasionally more) around a relatively constant mean. Since these light intensity changes could be detected with the eye, we are certain that they do not result from movements of the animal. A two-hour portion from such a recording is reproduced in Figure 1A. It can be seen that there is an instance when the light intensity doubled, apparently spontaneously. Such a marked increase in light intensity also occurs when the animal is handled. Indeed we found that striking the test tube in which the animal was placed would evoke such a response (Fig. 1B). The response still occurred immediately after the animal had been decapitated. Upon stimulation of the nerve cord which induced electrical shocks no luminescent response was observed which could be attributed to the effect of the electrical stimuli. The way in which the luminescent changes are brought about in the living animal is not clear.

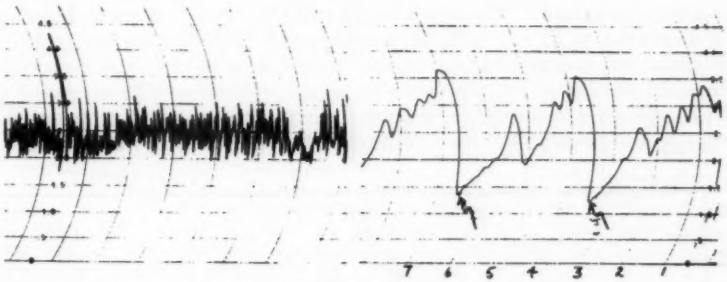

FIGURE 1. Reproductions of recordings of *Luminodesmus* luminescence. Ordinate, light intensity; abscissa, time, to be read from right to left in both cases. *Left:* Luminescence of an undisturbed animal over a two-hour interval. Time between vertical divisions, 15 minutes. *Right:* Luminescence changes of an animal where the test tube containing the animal was tapped lightly at the two instances noted by arrows. Time between vertical divisions, one minute.

Isolated pieces of the animal retain their luminescence for a long time. The intensity decreases gradually to about one half of its original value in 8 hours. Although fluctuations in intensity may occur for the first 15 minutes, it is essentially a steady luminescence thereafter. An eviscerated specimen from which the first few and last few segments are cut off behaves in essentially the same manner. Animals removed from humus and kept in a test tube for a day or more also showed little fluctuation in intensity. Whether or not this was due to water depletion or to starvation was not determined. Preparations with little or no light intensity fluctuation were used in the various experiments described below.

2. Possibility of luminous symbiotic bacteria

In some organisms luminescence arises from an association with luminous bacteria (see Harvey, 1952). The possibility that this might be the case in *Luminodesmus* was investigated some years ago by W. D. McElroy and one of us (J. W. H.). Whole animals and extracts of animals were put on agar plates containing a variety of media. Plates with a range of salt concentrations (0, 1%, 2% and 3% sodium chloride) were made up with both glycerol and glucose as carbon sources and Bacto Tryptone. No growth of luminescent bacteria occurred on any plate.

It may also be noted that the effect of varying oxygen concentration upon the luminescence of *Luminodesmus* (see section 5) indicates that the light is not bacterial in nature. In bacteria (Hastings, 1952; Shapiro, 1934) decreasing the oxygen concentration has no effect upon luminescence unless the concentration is less than about 0.3%.

3. Color of the light

The light emission from *Luminodesmus* is weak, requiring dark-adaptation on the part of the observer to see it. The emission spectrum was determined by placing a single animal at the entrance slit of a Bausch and Lomb Grating Monochromator, and the phototube at the exit slit. A second phototube was placed by the entrance slit to monitor any changes in the intensity of the animal during the course of the experiment. The entrance and exit slits were both set at 1 mm., which gives a dispersion of 12 mμ with the grating used (15,000 lines per inch). The light intensity was measured at the various wave-length settings and then corrected for phototube sensitivity and for monochromator efficiency. The corrected values are plotted against wave-length in Figure 2, giving the emission spectrum with the maximum in the green at 495 mμ. This spectrum is similar to that of some of the

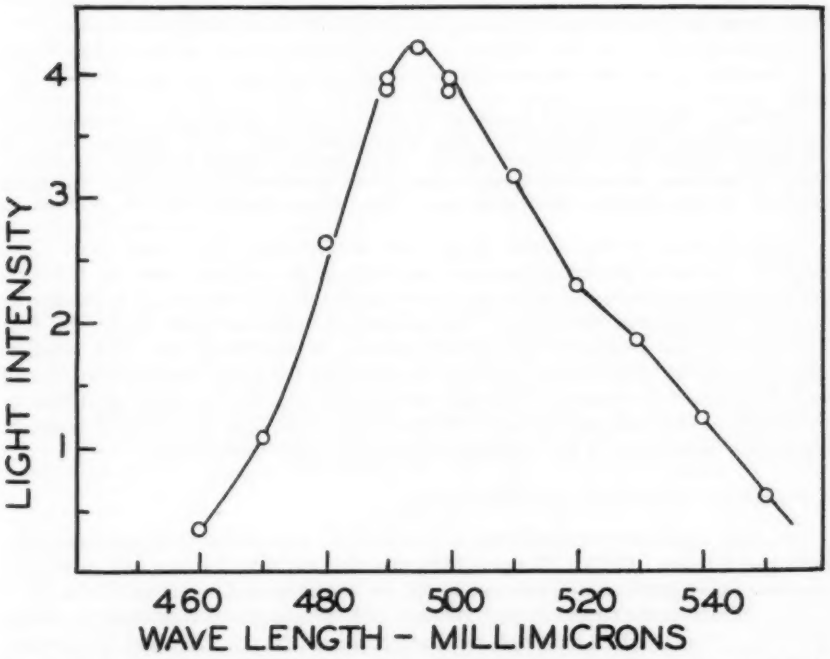

FIGURE 2. Emission spectrum of *Luminodesmus sequoiae*. Ordinate, light intensity in arbitrary units, corrected for phototube sensitivity and monochromator efficiency.

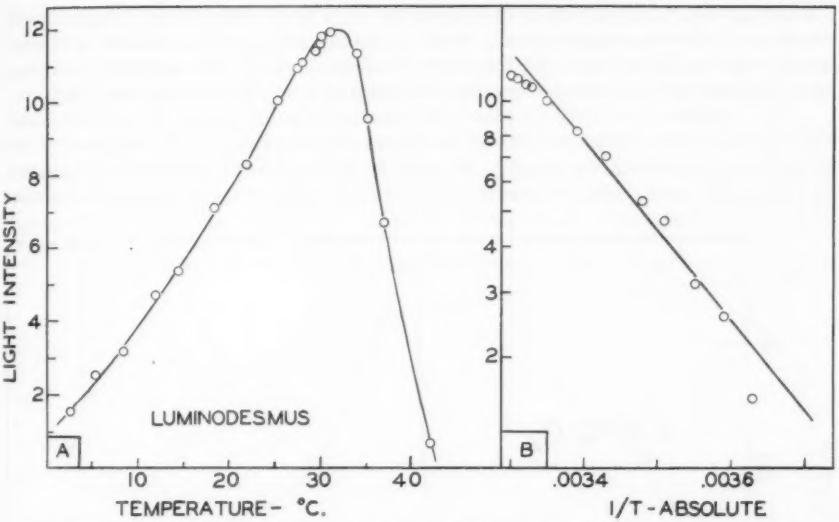

FIGURE 3. (A) Effect of temperature upon luminescence. Ordinate, light intensity in arbitrary units. See text for details. (B) Data of Figure 3A plotted according to the Arrhenius equation. Ordinate, light intensity in arbitrary units plotted on a log scale; abscissa, the reciprocal of absolute temperature.

luminous bacteria, although the spectrum of bacterial emission may depend upon the density of the suspension (Harvey, 1952).

4. Effect of temperature

The effect of temperature upon the luminescence was determined using both intact organisms and eviscerated specimens. The results were essentially the same in both cases. The specimen was held in place in a test tube by a cotton plug. Temperatures were adjusted by holding the tube in a water bath, and the temperatures plotted are those read from a thermometer placed in the tube beside the specimen. The tube was then quickly removed from the bath and placed in front of the phototube.

The data from one experiment with an eviscerated specimen are plotted in Figure 3, along with a plot of the data according to the Arrhenius equation. The Q_{10} for the process is 1.95 between 10° and 20°; 1.73 between 15° and 25°; and 1.55 between 20° and 30°. The sharp decrease in luminescence above the optimum of 31.5° is most likely the result of heat denaturation of enzymes. The activation energy for the over-all process may be calculated from the slope of the straight line drawn in the Arrhenius plot. The value obtained in this case is about 12,000 calories.

5. Effect of varying oxygen concentration

The effect of oxygen concentration upon the luminescence was determined quantitatively, using both whole animals and eviscerated specimens. Both gave

similar results. Luminescence was greatest in 100% oxygen and progressively decreased at lower concentrations, being reversibly extinguished in pure nitrogen, as reported by Davenport, Wootton and Cushing (1952). An oxygen concentration of about 6.5% decreased the intensity from that in air by about one half.

The specimen was held in place with cotton in a stoppered 10-ml. test tube, with glass tubing to bring the gas mixtures into the test tube. Gas mixtures of the desired oxygen concentration were prepared by mixing nitrogen with air or oxygen at measured rates, using calibrated flow-meters. The typical effect of lowered

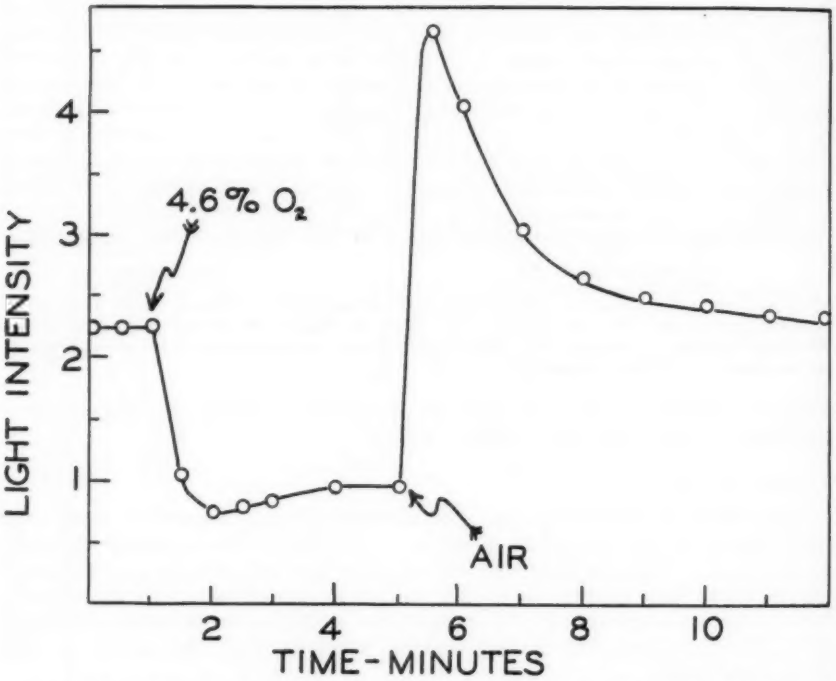

FIGURE 4. Changes in luminescence with time when a gas mixture containing 4.6% oxygen was passed over the animal for four minutes followed by the readmission of air.

oxygen concentration upon luminescence is shown in Figure 4. The response is essentially similar to that found in several other luminous organisms (Hastings, 1952; Hastings, McElroy and Coulombre, 1953; Hastings and Buck, 1956). There is a characteristic "undershooting" when the animal is exposed to a lowered oxygen concentration, and an overshoot or excess flash of luminescence, when it is exposed to a higher concentration. This excess luminescence is greater when a lower oxygen concentration is used during the period previous to the time when air is readmitted. For example, in the experiment shown in Figure 4, the luminescence was about twice the baseline level when air was admitted. With

1% oxygen the luminescence was 2.5 times and with 10% oxygen it was 1.5 times the baseline level. This suggests that the substrate for the luminescent reaction (luciferin) is the product of a series of relatively slow reactions. When the oxygen concentration is changed the luciferin comes to a new steady-state concentration, but only relatively slowly.

The values for light intensity versus oxygen concentration plotted in Figure 5 are the steady-state values, measured just previous to the time when the animal was returned to air. The data plotted are the results obtained with six different specimens. Although there was a variation in the results, the data for any given

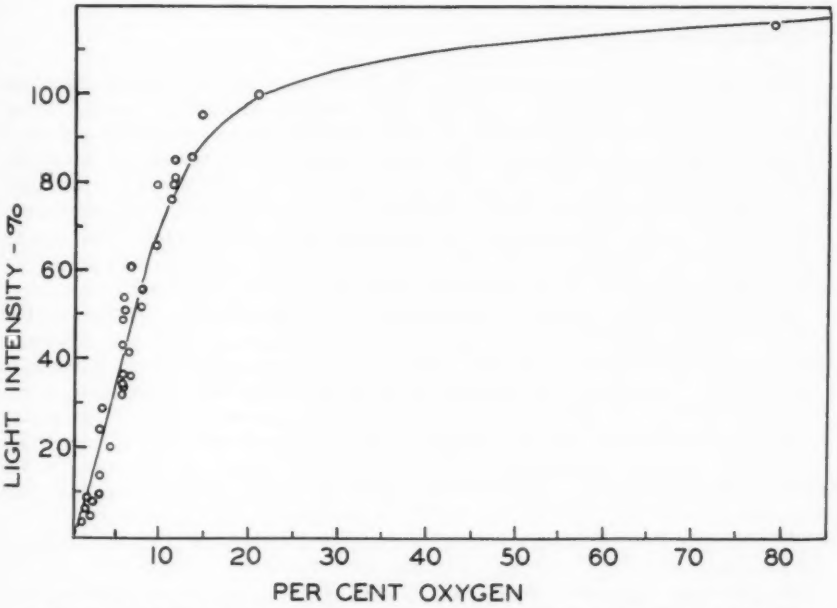

FIGURE 5. Effect of oxygen concentration upon the steady-state luminescence of *Luminodesmus*. Data are taken from experiments such as are illustrated in Figure 4. The luminescence, in low or high oxygen just prior to the readmission of air, is expressed as the per cent of the steady-state of luminescence in air.

animal fall along a smooth curve, suggesting a real difference between one animal and another, rather than error in the method. The reason for these differences could not be ascertained. Neither carbon monoxide nor carbon dioxide when added to the gas mixtures in 1% concentrations had any effect upon the shape of the curve for a given animal.

6. Fluorescence

Luminodesmus is highly fluorescent under ultraviolet light, and it was suggested previously (Davenport *et al.*, 1952) that this fluorescent compound might be in-

volved in the luminescent reaction. In luminous bacteria, for example, it has been demonstrated that reduced flavin mononucleotide ($FMNH_2$) is involved in the luminescent reaction, possibly as luciferin (McElroy, Hastings, Sonnenfeld and Coulombre, 1953; Strehler, Harvey, Chang and Cormier, 1954). FMN and other flavins are highly fluorescent in the oxidized form and are not at all fluorescent in the reduced form. Under anaerobic conditions in the intact animal the luciferin should be essentially 100% in the reduced state. However, there is absolutely no change in the fluorescence of *Luminodesmus* during anaerobiosis. The possibility that the fluorescence of the organism comes from a flavin compound involved in the luminescent reaction must therefore be ruled out.

7. Luminescence in extracts

Davenport, Wootton and Cushing (1952) reported negative results in all attempts to restore luminescence in filtered water extracts of *Luminodesmus*. Using sensitive light-measuring equipment, we have repeated their experiments and made additional studies with extracts. The most suitable method which we found for preparing extracts was to remove the gut from animals and dry them overnight in a vacuum desiccator with calcium chloride. The dry animals were then pulverized to a fine powder by grinding, and extracted with cold acetone. The acetone was removed by filtration and the dry powder kept in a vacuum desiccator. The powder remained active in this state for a period of at least two weeks.

When this powder was mixed with water a dim luminescence occurred (visible only to the dark-adapted eye), lasting for about 10 minutes, the half time for decay being about two minutes. Stirring always resulted in a temporary increase in luminescence, suggesting that leaching from particulate matter was taking place. Following stirring the light intensity returned to the original level. When such a solution was filtered through fine sintered glass (maximum pore size, 5.5 microns), the filtrate retained luminescence. Stirring did not affect the intensity of the filtrate, indicating that the particles from which leaching was occurring had been removed.

The intensity of luminescence in these extracts was dependent upon the pH of the solution, with an optimum at about pH 8.9. The determination was made by extracting equal quantities of the powder with 0.05 *M* trihydroxyaminomethane-maleic acid buffer at various pH values and measuring the light intensity of the solution. In all experiments described below a buffered extract at pH 8.9 was used, and buffered reagents where needed.

In the classical luciferin-luciferase test a fraction which has been extracted with hot water is combined with a cold water extract in which the luminescent reaction has been allowed to run to completion. In the hot water extract the enzyme has been destroyed, presumably leaving available substrate, or luciferin. In the cold water extract the luciferin has all been used up leaving active enzyme, or luciferase. The two mixed together should therefore give light, but with *Luminodesmus* completely negative results were obtained. Moreover, neither the hot water extracts nor exhausted cold water extracts, when added to a luminescing extract, had any effect upon the light intensity.

Attempts to separate the presumed luciferase and show its activity in the reaction were negative. Fractions were obtained by ammonium sulfate precipitation,

by alcohol fractionation, and by dialysis. None was active when added either to luminescing mixtures or to hot water extracts.

A large number of compounds were tested for their ability to modify the light intensity in luminescing extracts.[2] ATP was found to have appreciable activity. ATP added to an extract which was emitting light caused the intensity to increase by 10 to 30 per cent. The effect was not due to a pH change since buffered ATP solutions were used. It was effective with filtered extracts as well as unfiltered. Other pyrophosphate compounds were not tried, so it is possible that the action of ATP could be non-specific, similar to the effect of pyrophosphate compounds added secondarily to luminescing firefly extracts (McElroy, Hastings, Coulombre and Sonnenfeld, 1953). The fact that ATP would not restore luminescence to dark extracts indicates such a non-specific role. No restoration of luminescence occurred even when ATP was added to hot water extracts together with exhausted cold water extracts. However, when $MgSO_4$ was added following ATP addition, there was an additional increase in light intensity, suggesting the possibility of a more specific role for ATP. Also, 0.05 *M* Versene (ethylene diamine tetra acetic acid) was found to depress luminescence, indicating the possibility that the reaction is activated by a metal ion. All of the coenzymes listed in footnote 2 were tested in combination with ATP. None was found to have any stimulatory effect, although FMN and riboflavin slightly depressed luminescence.

The results give little clue as to the nature of the reaction. If a luciferin type compound is involved in a classical oxidative reaction, possibly in combination with ATP, then the luciferin must be highly unstable. In fact, such a highly unstable and heat-labile luciferin could account for the results we have obtained. The inhibition by flavins might mean that some flavin compound is involved in the reaction, although we would not expect a flavin to be particularly unstable.

Summary

1. The luminescence of *Luminodesmus* is continuous, but fluctuates by 20 to 40 per cent or more. The mechanism by which light emission is controlled is not known. No evidence was found for the suggestion that the light is bacterial in origin.

2. The luminescence is green with a maximum emission at 495 mμ and is optimal at a temperature of 31.5° C. Light emission is greatest in pure oxygen and extinguished in pure nitrogen. An oxygen concentration of 6.5% decreased the intensity from that in air by about one half.

3. Luminescence in water extracts of dried acetone powders has been demonstrated.

4. We have not been able to restore luminescence to dark extracts either by the classical luciferin-luciferase technique or by adding a variety of biochemical inter-

[2] Substances tested for activity, either singly or in combination, were: adenosine triphosphate (ATP), $MgSO_4$, riboflavin, flavin mononucleotide (FMN), flavin adenine dinucleotide, oxidized and reduced diphosphopyridine nucleotide, oxidized and reduced triphosphopyridine nucleotide, coenzyme A, beef heart extract (Armour), yeast concentrate (Sigma), liver concentrate (Sigma), do-decyl aldehyde, ethyl alcohol, glycerol, glucose, glucose-1-phosphate, thiomalate, thiooctate, glutathione, cystine, cysteine, KCN, sodium arsenite, iodoacetate, sodium fluoride, sodium azide, p chloro-mercuro-benzoate, naphthoquinone, quinhydrone, hydroquinone, quinone, firefly extracts, NaCl, Na_2HPO_4, KH_2PO_4 and $MnSO_4$.

mediates. We have found that if adenosine triphosphate is added to extracts while they are luminescing an increase in light intensity occurs.

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THE ANTIMITOTIC AND CARCINOSTATIC ACTION OF OVARIAN EXTRACTS[1]

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The search for chemical substances which might have a retarding effect on the growth of tumors has led along many paths. All sorts of substances have been tried. Those investigators who have theorized at all have for the most part thought in terms of some block of metabolic activity. Our own program has sought to discover relatively non-toxic antimitotic substances of natural origin, and in the search for such substances we have based our attack on what we believe to be a proper theory for the initiation and suppression of mitosis. Such a theory is discussed in some detail in a recent book (Heilbrunn, 1956). It holds that the mitotic spindle results from a gelation of the protoplasm, the mitotic gelation. Various substances can prevent this gelation by keeping protoplasm fluid. And because the most usual type of protoplasmic gelation and the type involved in the mitotic gelation is a clotting similar to the clotting of blood, it is our belief that anticlotting agents such as heparin or similar substances can prevent cell division. This indeed they do. The protoplasmic colloid contains substances which favor clotting and those which tend to prevent it. We have made extracts from various tissues and have found that ovaries are especially rich in anticlotting agents. These appear to resemble heparin and to be mucopolysaccharides. This work (Heilbrunn, Wilson and Harding, 1951; Heilbrunn, Chaet, Dunn and Wilson, 1954; Heilbrunn and Wilson, 1956) showed that the ovaries of various invertebrates and fishes do actually contain antimitotic substances which prevent the mitotic gelation and suppress cell division. In the search for some substance or substances which might eventually prove to have clinical value, we have recently investigated the ovaries of mammals and especially large mammals. In what follows, we will attempt to show first that extracts of mammalian ovaries do have antimitotic action, an action which is associated with a liquefying or anticlotting effect on the protoplasm; and second that such extracts may possess carcinostatic activity.

Materials and Methods

In studying antimitotic activity, the most favorable test objects and the easiest to work with are the eggs of various marine invertebrates. Eggs such as sea urchin eggs or those of the marine worm Chaetopterus divide synchronously following fertilization. These eggs can be obtained in large quantity and they represent a surprisingly constant material. In previous studies on Chaetopterus eggs, we have described the simple techniques required (Heilbrunn and Wilson, 1948). Most of

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our work was done on Chaetopterus eggs. One experiment was done on eggs of the clam Spisula. The technique required for the use of this egg has been described by Allen (1953). All of our experiments were done at a controlled temperature of 21° C. Two minutes after insemination the eggs were placed in the solutions to be tested.

Crude extracts were prepared by cutting the ovaries into thin slices and immersing these slices in solutions of acidified sea water at a pH of approximately 4.8. For each gram of tissue, one ml. of sea water was used. After extraction the extract was brought to the pH of sea water by the addition of NaOH. In a few cases the ovaries were homogenized before extraction, but this seemed to produce less favorable results in our antimitotic studies. The acidification of the sea water used in extraction was apparently not necessary and potent antimitotic extracts could be obtained over a wide range of pH values.

The Chaetopterus egg has a very fluid protoplasm which stiffens markedly before the formation of the mitotic spindle. There is indeed a two-fold increase in the viscosity of the protoplasm and this can readily be followed with the centrifuge method described in earlier studies. With a simple hand centrifuge, turned so as to give a force approximately 2250 times gravity, for some time after fertilization it requires only 7 seconds to move granules through the protoplasm of the Chaetopterus egg to such an extent as to give the appearance of zones. The number 7 is taken as a relative value for the viscosity of the protoplasm; actually it probably represents about twice the value of the viscosity in centipoises. At approximately 30 minutes after fertilization, 14 seconds of exposure to a force 2250 times gravity is necessary to produce the appearance of zones in the protoplasm. In studying the antimitotic and the anticlotting action of our extracts we determine the percentages of cleavage following exposure to various dilutions, and we also determined the effect of the extracts on the viscosity of the protoplasm at the time when the normal control eggs showed increased viscosity. These tests had to be made rapidly, for the duration of the mitotic gelation is short.

Crude extracts prepared in the way we have indicated are very potent anticlotting and antimitotic agents, as our results will show, but generally speaking they are not very effective as carcinostatic agents. Indeed these extracts contain not only substances which tend to prevent protoplasmic clotting, they also contain substances which have exactly the opposite effect. Thus, although in one case we did obtain a definite carcinostatic effect with such crude extracts, for the most part we were not successful, and indeed in some instances the survival time of cancerous mice treated with crude extracts was decreased rather than increased. Hence we were led to try and find methods of extraction which would give us preparations of as high a carcinostatic action as possible with a minimum of toxicity or adverse action.

For many months, we had little or no success. Then we hit on a method of fractional alcoholic precipitation, and this has now provided us with extracts which possess a definite carcinostatic action. This was perhaps to be expected, for in studying the antimitotic action of extracts of starfish ovaries, we found that the potent substance could be precipitated by alcohol (Heilbrunn, Wilson and Harding, 1951).

The procedure we finally adopted was the following: Cow ovaries, fresh from the slaughter-house, were ground up in a meat grinder and were then extracted

in a solution containing 0.9% sodium chloride and 0.125% sodium bicarbonate. For each gram of ovarian material, 2 ml. of solution were used. The extraction was carried out in a cold room at 5° C. with constant stirring, and was continued for 16–18 hours. After the extract was strained through cheesecloth, it was centrifuged at 2000 rpm in a refrigerated centrifuge. The supernatant was then centrifuged in a Spinco centrifuge at 16,000 rpm (20,000 g) for an hour. The resultant supernatant was then precipitated by various concentrations of alcohol at approximately 0° C. Following each precipitation by a given percentage of alcohol, the supernatant was decanted. The precipitates were then lyophyllized. Our final product represented only a small fraction of the original ovarian material. Thus typically by precipitation with 45–60% ethanol we obtained about 10 mg. from a kilogram of ovaries.

In studying carcinostatic action, we used Swiss white mice. These were inoculated routinely with 1,500,000 Ehrlich ascites tumor cells in a volume of one ml. of ascitic fluid. Then 24 hours after inoculation, treatment with the extracts was begun. Each day for five days, each one of the mice to be treated was injected with a solution containing 15 mg. of the material. In general the 15 mg. were dissolved in 0.5 ml. of saline solution. Details of our technique and growth studies of our particular tumor will be published in a paper to be written by two of us (Tosteson and Davidson).

Results

In the summer of 1955, we tested the effect of crude extracts of mammalian ovaries on cell division in the Chaetopterus egg. These extracts in every case stopped cell division. They also prevented the mitotic gelation. Our results are shown in Table I. In most cases the viscosity of the protoplasm at 30–40 minutes after fertilization is given as "less than 8." It may have been decidedly less than 8, but in the short space of time available for these measurements it was not possible to make enough tests to be sure of an exact value. However, the fact that the viscosity was less than 8 is a sure indication that the mitotic gelation has been suppressed, for during this gelation, the viscosity rises to a value of 14.

In addition to the data presented in the table, we have results from a few additional experiments; these results are entirely consistent with those shown in the table. In most of these other experiments, we attempted to improve the potency of the extracts by purifying them, but in every case the crude extracts prepared as described in the previous section were superior to the "purified" product. We also tried modifying our extraction procedures. In the results reported in the table, the extractions were made at a pH of 4.8, but we also tried extracting the ovarian material at pH's of 2.25, 3.90, 6.15, 7.1, and 10.0. All of the extracts prepared at these different pH's were likewise effective. Heating the extracts did not seem to have a very harmful effect, although our results were somewhat variable and minor differences in procedure seemed to be important. In one experiment an extract kept at 100° C. for three hours was still highly potent. In other experiments, exposure to 100° C. for shorter periods caused some loss in potency. Extracts made in distilled water and then lyophyllized were not effective. When the ovaries were homogenized before being extracted, the results were less favorable. Homogenization seemed to favor the release of thromboplastic substances into the extraction medium.

Although during the summer of 1955, we were never able to obtain any degree of successful purification of our extracts, in the following winter one of us (R. J. Rutman) hit upon the plan of precipitation with various concentrations of alcohol. Some of the precipitates obtained in this way when dissolved in saline solution had a very definite carcinostatic action, and we determined therefore to test the antimitotic action of these precipitates. Chaetopterus eggs (at Woods Hole) are only available during the summer months, so that we had to wait until the summer of 1956 before making our tests.

In making these tests, we had some difficulty, for the precipitates dissolved scarcely at all in the sea water in which we had to use them. However what tests we were able to make with the limited amount of material we had at our disposal indicated that only those precipitates obtained with intermediate concentrations of alcohol were effective. We had three fractions, of which A represented the precipitate obtained from cow ovary extracts with 0–45% alcohol, B the precipitate obtained with 45–60% alcohol, and C the precipitate with 60–80% alcohol. Of these three fractions, neither A nor C was very soluble. Fraction B seemed to go into solution, but when the solution was looked at under the microscope it was seen to have a large number of small solid particles suspended in it. We did several experiments with this fraction B. In one of these experiments, 5 mg. were dissolved in one ml. of sea water and then this solution was diluted so that the resultant dilutions contained 2.5 mg. per ml. of sea water and 1.25 mg. per ml. of sea water, respectively. All three of these solutions prevented the mitotic gelation and in all of them the fluidity of the protoplasm of the Chaetopterus eggs was maintained. In the most concentrated solution only 29% of the eggs cleaved, in the middle concentration 19%, and in the most dilute of the three solutions, 26% of the eggs cleaved. In the control 90% of the eggs cleaved. These counts were made 30 minutes or more after 50% of the control eggs had cleaved. The fact that the

TABLE I

Effect of ovarian extracts on the mitotic gelation and on cleavage of the eggs of Chaetopterus

Source of material	Duration of extraction in minutes	Dilution	Viscosity at 30–40 min.	% cleavage	% cleavage in control
Cow	30	—	4	1	98
Cow	60	—	4	0	98
Cow	120	—	less than 8	0	100
Cow	120	$\frac{1}{2}$	less than 8	0	100
Cow	120	$\frac{1}{4}$	about 8	0	100
Cow	120	$\frac{1}{8}$	about 8	5	100
Cow	60	—	less than 8	0	99
Cow*	60	—	5	0	99
Cow**	60	—	5	0	99
Cow	120	—	less than 8	0	99
Cow	120	—	less than 8	0	100
Pig	155	—	less than 8	0	99
Lamb	105	—	less than 8	0	99

* Ovarian capsules.
** Corpora lutea.

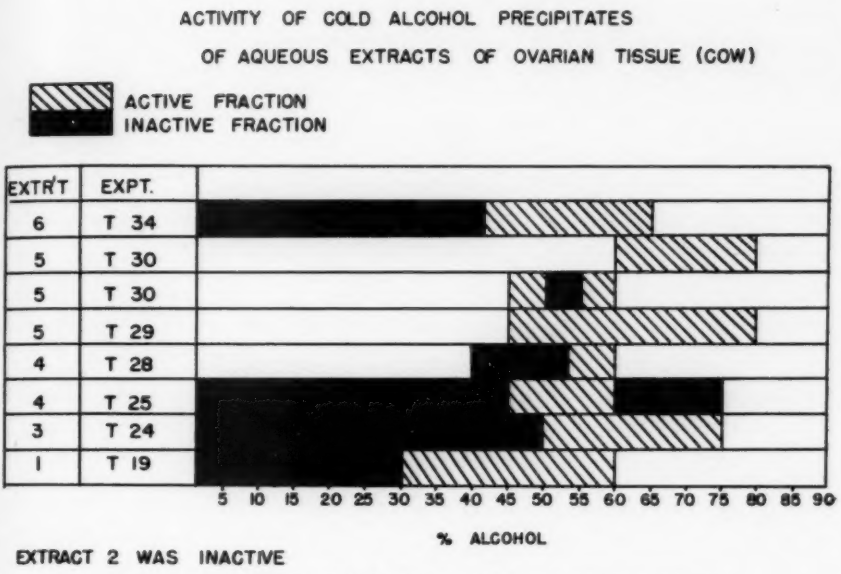

FIGURE 1. Activity of cold alcohol precipitates of aqueous extracts of ovarian tissue (cow).

various concentrations of material all acted in essentially the same way indicates that the material was only slightly soluble so that the actual concentration in true solution was the same in all three cases. In the very few experiments that we did with fractions A and C, these were without effect, perhaps because their solubility was extremely low.

In addition, the material of fraction B was tried on the eggs of the clam Spisula, and in this case also the material exerted an antimitotic effect. Concentrations of 2.5 mg. per ml., 1.25 mg. per ml. and 0.625 mg. per ml. showed strong antimitotic action, the cleavage being reduced from a control value of 97% to values of 31% for what presumably was the more concentrated solution, 39% for the intermediate concentration, and 40% for the weakest concentration.

Thus in spite of the fact that the material was soluble only slightly, the fraction obtained by precipitation with 45–60% alcohol did actually exert an antimitotic action, and in the case of Chaetopterus eggs this action was associated with a prevention of the mitotic gelation. No attempt was made to study the effect of fraction B on the mitotic gelation in the Spisula egg, for as yet the cycle of viscosity changes in the Spisula egg has not yet been worked out with sufficient thoroughness.

Clearly, our results with marine eggs indicate that fractionation of the crude cow ovary extracts by alcoholic precipitation can preserve the antimitotic and antigelating action.

Let us consider now the carcinostatic action of these alcoholic precipitates obtained from extracts of cow ovaries. Up until the present, we have accumulated a

large body of data. Indeed we have experimented with well over 4,000 mice. Our results show that the precipitates obtained by treating cow ovary extracts with intermediate concentrations of alcohol can cause survival of some 15–25% of mice previously inoculated with a lethal tumor, a tumor which regularly kills 100% of all mice properly inoculated with it.

Figure 1 illustrates the fact that treatment with intermediate concentrations of alcohol in the cold can produce precipitates which have a carcinostatic action. This figure gives the results obtained with five different extracts and is based on experiments with about 2,000 mice. Further details of these experiments will be presented in another paper soon to be prepared by several members of our group (Tosteson, Davidson and Rutman). Since the data for the table were collected we have obtained additional confirmatory data. Also we have been experimenting with various other types of extraction media and with other types of fractionation. Preliminary results lead us to the hope that extracts and fractions can be obtained which will cause a higher percentage of survival than we have been able to obtain with the extracts described in this paper.

Discussion

Our work has been based on the idea that the protoplasm of all cells is much alike both chemically and physically, that inasmuch as the process of mitosis is much the same throughout the animal kingdom, the forces and agents which initiate cell division and those which suppress cell division are also much the same. On the basis of this pattern of thought, and on the basis also of many experiments on the initiation and suppression of mitosis (see Heilbrunn, 1956), we have been able to develop a new type of carcinostatic agent. At present this agent is at least as potent for our Ehrlich ascites tumors as other long-studied agents. It is our hope that the type of agent we are using can be perfected to give even better results. This will require much additional work.

Summary

Extracts of the ovaries of cows, pigs and sheep can suppress mitosis in eggs of the worm Chaetopterus. This they do by keeping the protoplasm fluid and inhibiting the mitotic gelation which is a necessary precursor of the mitotic spindle. The potent substance or substances in extracts of cow ovaries can be precipitated by treating the extracts in the cold with intermediate concentrations of alcohol. Such purified preparations have a definite antimitotic effect and they also have a very definite carcinostatic action.

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CARDIAC PHYSIOLOGY OF THE SCORPION PALAMNAEUS BENGALENSIS C. KOCH [1]

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The nature of heart-beat among arthropods has been studied by several workers (Prosser, 1942; Needham, 1950; Krijgsman, 1952). It has been observed in Crustacea and Insecta that the origin of heart-beat in each class is of varied types and has no relationship with the taxonomic classification. Among the arachnids, *Limulus* has a myogenic heart-beat in the young which becomes neurogenic in the adult (Prosser, 1942; Krijgsman, 1952) and the heart-beat of spiders is neurogenic (Rijilant, 1933). Even though Police (1902) indicated the presence of an epicardiac nerve on the heart of scorpion, the heart-beat of the scorpion, *Palamnaeus bengalensis* has been reported by Kanungo (1955) to be myogenic. It is of interest to find that, also in Arachnida as in Crustacea and Insecta, the nature of heart-beat is not of a single type. The hearts of arachnids are poorly understood and the present work is a detailed study of the physiology and pharmacology of the heart of *Palamnaeus bengalensis*.

Materials and Methods

Scorpions freshly collected from their natural habitat (Lal and Kanungo, 1953) were used for these experiments. They were lightly chloroformed and immediately dissected in a saline containing sodium chloride, 0.65 gm.; potassium chloride, 0.03 gm., and calcium chloride, 0.03 gm.; in 100 ml. of distilled water. The saline, which was prepared fresh before the experiments, was maintained at pH 6.3 using phosphate buffer, as the haemolymph of the scorpion was found to be on the acid side of neutrality in agreement with Maluf's (1939) statement. The heart was exposed fully *in situ* by carefully cutting the terga at the sides and removing them. Isolated heart preparations were made in petri dishes containing the saline. Effect of pH, temperature and drugs on the heart-beat were observed on hearts both *in situ* and isolated. Nearly 150 heart preparations have been made for various drug experiments.

Anatomy of the Heart

Like that of *Buthus* (Parker and Haswell, 1940), the heart of *P. bengalensis* is eight-chambered, spongy and muscular. It is enclosed in a pericardium and is

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held in its position between the two lobes of the liver by eight pairs of alary muscles. It is 2.5 cm. long in a medium-sized scorpion with a body length of approximately 5 cm. A thin-walled anterior aorta arising from the heart bifurcates on the oesophagus. A thin-walled posterior aorta proceeds to the tail.

General Properties of the Heart-Beat

Hearts both *in situ* and isolated showed a high degree of automaticity; contractions occurred simultaneously throughout the myocardium and following one another in a regular uninterrupted sequence rhythmically. Simultaneous contraction of the scorpion heart had been reported earlier (Du Buisson, 1925). When first isolated, the rate of beat of the heart was irregular and slow, but it became normal after about 5 minutes, and showed a little acceleration in the rate of beat as compared with that of hearts *in situ*. The rate of beat of intact hearts was 50–54/minute at room temperature (25–27° C.). Cutting of the alary muscles *in situ* resulted in a slight increase of the rate to 56–62/minute, which was the same as that of isolated hearts. It appears, therefore, that even though the property of automatic movement lies in the muscles of the heart itself, the regulation of the rate of beat is effected by alary muscles. The rate of isolated heart preparations remained normal for 10–12 hours after which it decreased and the amplitude fell gradually. Mechanical stimuli like shaking the saline or pressing the heart with a needle temporarily inhibited the heart-beat. After 2–4 minutes, an acceleration in the rate was observed. Excised pieces of the heart beat for about 4–5 minutes. In all preparations, the anterior end stopped first and the posterior end later, suggesting that the pace-maker of the heart is situated at the latter end.

Course of Circulation

As stated above, isolated hearts beat with the anterior and posterior ends contracting and relaxing simultaneously. During diastole the heart shortens in length and bulges and the haemolymph flows in through the paired ostia. During systole, the heart extends lengthwise, the ostial valves close and the haemolymph is expelled at both the ends. A freshly isolated heart was placed in a dry watch glass in such a manner that the two ends were at a higher level than the middle region. A drop of neutral red was put at the center of the watch glass. The heart continued to beat and neutral red was seen flowing out at both the ends.

Effect of Temperature

Isolated hearts in petri dishes containing the saline were kept at different temperatures in an incubator and their rates were noted. An upper limiting rate of 80–85/minute was observed at 42° C., above which beating ceased permanently. The lower limiting rate on cooling was 4–5/minute at 5° C. Below this temperature the heart ceased beating but recovered when the temperature was increased.

Effect of pH

Separate stocks of the same saline solution were prepared by buffering with phosphate buffer between pH 5.5 and pH 7.5 and the hearts were kept in these

salines. The heart remained active between pH 6.1 and pH 6.5. With increase or decrease of the pH of the saline beyond this range, depression of the heart rate occurred.

Effects of Drugs

Fresh dilutions of 10^{-3}, 10^{-4}, 5×10^{-4}, 10^{-5}, 5×10^{-5}, and 10^{-6} of various drugs were made in the saline before each set of experiments. Both intact and isolated hearts were bathed side by side with one of the diluted drugs to compare their effects on the heart-beat in isolated and *in situ* preparations. No difference between isolated and intact hearts was observed. Mechanical shock to the heart was avoided as far as possible. The drug was sucked out with a pipette, the heart

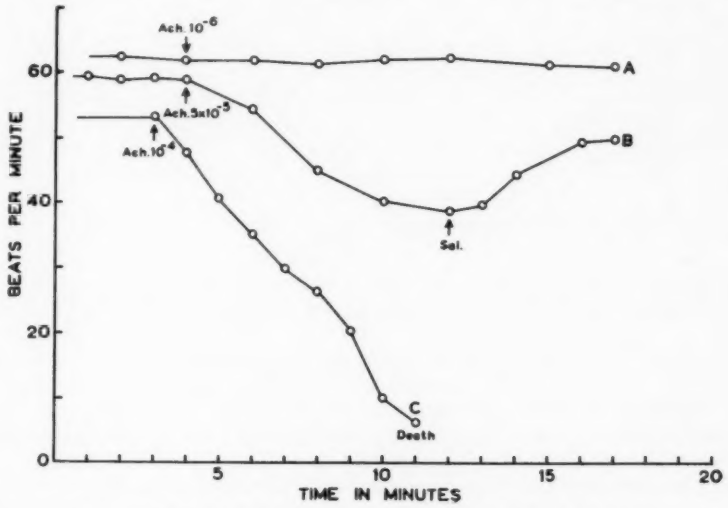

Figure 1. Effect of acetylcholine on the heart of *P. bengalensis*. Ach., acetylcholine; Sal., saline.

washed with the saline three times and the fresh drug added slowly. Recordings of the heart rate were made following the methods of Jones (1954). Three recordings, one minute each in length, were made one minute after adding the drug.

Acetylcholine more dilute than 5×10^{-5} had no effect on the heart rate. Concentrations of 5×10^{-5} or stronger depressed the heart rate; the time taken for depression was inversely proportional to the concentration of the drug (Fig. 1). There was a gradual weakening of the strength of beat, reduction in the amplitude, rest-pauses and sporadic irregularities in 5×10^{-5} or stronger concentrations. In no case was there any acceleration before the depression. Neither did the beat recover if the heart was left in the drug. However, all such hearts recovered after they were washed with the saline, but the normal rate of beat was never reached. Some hearts showed tolerance to the drug up to 10^{-4} after prior treatment with more dilute solutions and gradually increasing concentrations (Fig. 2D).

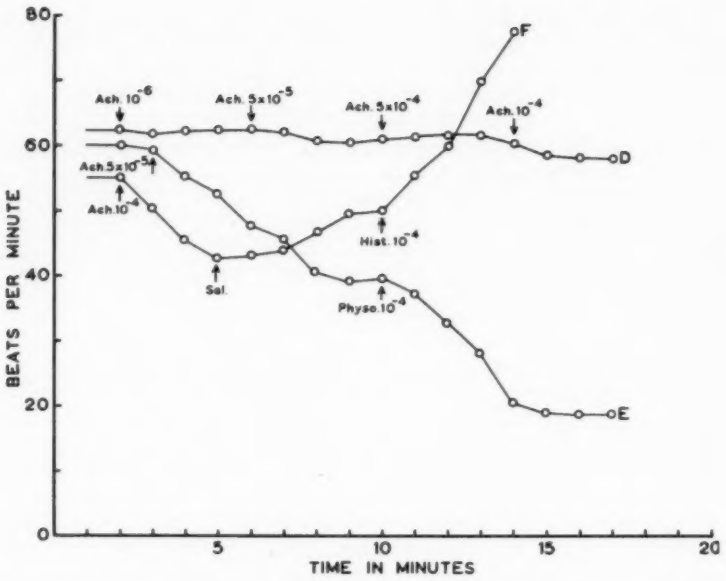

FIGURE 2. Effects of various drugs on the heart of *P. bengalensis*. Ach., acetylcholine; Adr., adrenaline; Atr., atropine; Hist., histamine; Physo., physostigmine; Sal., saline.

Physostigmine at 10^{-4} or stronger did not by itself show any appreciable effect on the heart rate. However, application of acetylcholine to the heart, after treatment with physostigmine, potentiated the effect of acetylcholine (Fig. 2E).

Histamine at 10^{-4} or stronger accelerated the heart rate to a maximum of 85/minute and this effect was reversible on washing with the saline. The time taken for the heart to reach the maximal rate in different dilutions was directly proportional to the dilutions of the drug. It antagonized acetylcholine action, and hearts collapsing under the treatment with acetylcholine could be revived by this drug. Such hearts also beat at 85/minute (Fig. 2F).

Adrenaline at 10^{-5} or stronger accelerated the heart rate to about 75/minute and this effect was reversible.

TABLE I

Comparison of the effects of drugs on the hearts of Limulus and P. bengalensis

	Ach.	Atropine	Adrenaline	Ether	Histamine	Physostigmine	Chloroform
*Limulus**	+	+	+				
P. bengalensis	−	−	+	0	+	0	0

+, excitation; −, inhibition; 0, no effect.
* Krijgsman, 1952.

Atropine at 5×10^{-4} or stronger inhibited the heart rate reversibly.

Half-saturated and fully-saturated aqueous solutions of *ether* had no observed effect on the heart-beat.

Chloroform had no observed effect on the heart-beat.

It was found in all the cases that the drug-treated hearts recovered after washing with the saline. A quicker recovery of the heart was attained by using warm saline which was added slowly to the heart container. The time taken for such recovery varied from five to fifteen minutes.

Table I gives comparatively the effects of various drugs on the hearts of *Limulus* and *P. bengalensis*. Even though Table I does not indicate the effect of ether on the neurogenic heart of *Limulus*, it may be mentioned here that ether inhibits neurogenic hearts in low concentrations (Needham, 1950).

Haemolymph Pressure

Bleeding occurred when incisions were made at pedipalpi, abdomen and tail regions; this indicates positive haemolymph pressure throughout the body. By inserting capillaries in continuation with U-tubes, actual pressure was found to be 6 mm. of saline at the pedipalpi and at the abdomen.

Discussion

The pharmacology of the scorpion heart resembles that of the crustacean, *Daphnia* (Baylor, 1942) and the vertebrates, and has no resemblance to that of *Limulus*. According to Prosser *et al.* (1950), the hearts of arthropods are of non-innervated myogenic, innervated myogenic and neurogenic types, which description is based mainly on acetylcholine effect. Needham (1950) classified the crustacean hearts into two categories, myogenic and neurogenic, by taking several factors into consideration. The heart of *P. bengalensis* in showing (1) autonomous rhythmicity with contractions developing simultaneously throughout the myocardium, (2) insensitiveness to ether, and (3) inhibition by acetylcholine indicates that the nature of its beat or that of its pacemaker is innervated myogenic. The epicardiac nerve reported by Police (1902) appears to be either extrinsic or regulating in function.

Summary

1. The heart of *P. bengalensis* beats continuously at a rate of 50–62/minute at a temperature of 26° C. The contraction is developed simultaneously throughout the muscle.

2. Acetylcholine and atropine depress the heart-beat and their actions are reversible. Physostigmine potentiates the effect of acetylcholine.

3. Ether and chloroform have no effect on the heart-beat.

4. Histamine and adrenaline accelerate the heart-beat and their effects are reversible on washing with saline.

5. The haemolymph pressure is 6 mm. of saline.

6. It is concluded that the pace-maker of the heart of *P. bengalensis* is of the innervated myogenic type.

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A COMPARATIVE STUDY OF THE CUTICULAR STRUCTURE OF THREE FEMALE MEALY BUGS (HOMOPTERA: PSEUDOCOCCIDAE)

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The recent discovery in the arid north-west of South Australia of a new species of the hitherto monotypic genus, *Epicoccus*, afforded an opportunity to examine the cuticle of a drought-resistant pseudococcid. Members of the Pseudococcidae are normally confined to microhabitats where they are surrounded by humid equable conditions, and it was anticipated that considerable modification of cuticular structure would be shown by a species which has evolved in an area where it is fully exposed to the desiccating effects of high temperatures, low relative humidities, and drying winds. The extent of such modification, if any, could be gaged only after the cuticle had been compared with that of a typical form, of which, however, there appears to be no published account. A study of the cosmopolitan long-tailed mealy bug, *Pseudococcus adonidum* L., was therefore undertaken as a preliminary step in the investigation. Finally, the cuticle of the one described species of Epicoccus, *E. acaciae* (Maskell) was examined to ascertain whether any major differences in cuticular structure occur within the genus.

The terminology is in accord with the scheme which I recently outlined (Lower, 1956).

Materials and Methods

Specimens of *P. adonidum* were obtained locally from a heavily-infested plant of *Daphne odora*. Those of *Epicoccus* sp., were collected from *Acacia aneura* F. Muell., at Yudnapinna in the north-west of South Australia while those of *E. acaciae* came from the coastal strip of Western Australia, the only area in which the species is known to exist.

The insects were first killed with cyanide, and free-hand sections of some of each species at once stained with Sudan black B. The remainder, after fixation in Sanfelice's fluid, were embedded, part in a water-soluble wax and part in paraffin. Sections cut from these were similarly stained. Comparison showed that there was no observable loss of cuticular lipoid when paraffin was used as the embedding medium. All work was therefore done using paraffin sections cut at 4 μ and 1 μ, except that when the external wax of *Pseudococcus* was being investigated, sections prepared by the first two methods were used.

The histochemical tests and techniques applied have been described elsewhere (Lower, 1957a).

I. The Cuticle of Pseudococcus adonidum L.

The cuticle (Fig. 1) is thin, measuring in most parts between 6 μ and 8 μ; only exceptionally does it attain a thickness of 10 μ. Its structure is relatively un-

specialized and except for the brown outer layer of the epicuticle it is unpigmented. Pore canals, if present, could not be observed with the light microscope either under bright-field or phase-contrast conditions. A thick layer of wax (discussed later) covers the cuticular surface.

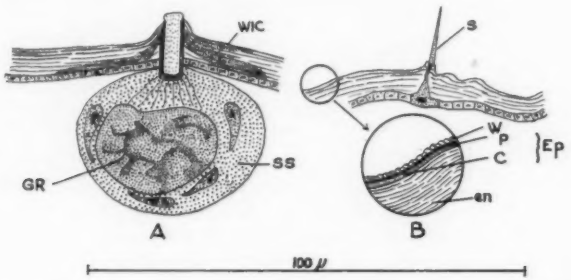

FIGURE 1. *P. adonidum:* Cuticle. A. Part of cuticle adjoining glandular duct. B. General cuticle. c, cuticulin layer; en, endocuticle; Ep, epicuticle; GR, gland reservoir; P, paraffin layer; S, spinule; SS, secretory sheath; W, surface wax layer; WIC, wax-impregnated cuticle.

A. The Epicuticle

The epicuticle (Fig. 1B) is two-layered and has a total thickness of about 1 μ, of which the inner layer constitutes the greater part.

The inner or cuticulin layer is colorless and transparent. It responds positively to the Millon, xanthoproteic and biuret tests, is non-argentaffin, and is unaffected by either Sudan black B or Nile blue sulfate. It is stained red by the routine stain and pink by Sevki's diluted Giemsa technique, but it cannot be stained either by Mallory's PTAH or any of the iron haematoxylins. Schmorl's test shows the absence of reducing substances. It is soluble in warm concentrated solutions of potassium hydroxide or hydrochloric acid. Its location and general chemical reactions indicate that it is probably homologous with the cuticulin layer of *Rhodnius* (Wigglesworth, 1947).

The thinner outer layer is brown-pigmented. The color is difficult to discharge, sections requiring about a week's immersion in 10% hydrogen peroxide to effect this. The strong positive response to Schmorl's test, together with the non-argentaffin nature of the layer, suggests that the coloring matter is a lipofuscin. The layer responds to none of the protein tests. It is blackened by Sudan black B, and stained deep blue by Cain's Nile blue sulfate technique. Prolonged differentiation during the latter process removes much of the blue without any red appearing so that acidic lipoid only appears to be present. It is stained black by iron haematoxylins and deep violet by Mallory's PTAH. It is resistant to concentrated solutions of potassium hydroxide and hydrochloric acid but fuming nitric acid or *aqua regia*, when gently warmed, attacks some of its constituents and liberates a material which is practically instantaneously soluble in cyclohexane and is quickly and easily stained by any of the fat stains. Its anatomical position and general chemical behavior indicate that the layer is a paraffin epicuticle (Dennell and Malek, 1955). It differs from the corresponding layer in Sarcophaga (Dennell,

1946) by being non-argentaffin, in giving no response to the xanthoproteic reaction, and in its content of lipofuscin pigment.

B. The Procuticle

Practically the whole of the procuticle is present as endocuticle. With the exception of the spinules mentioned below, no exocuticle occurs, while the mesocuticle is confined to the alveoli of the spinules and the small convex circular patches (appearing crescentic in transverse section) where muscles are inserted in the cuticle.

Scattered over the cuticular surface are weakly-sclerotized, colorless spinules (Fig. 1B), each secreted by a trichogenic cell which is much larger and more granular than the adjacent hypodermal cells. A cytoplasmic process from each trichogenic cell, after passing through the cuticle, attenuates and forms a core to the spinule for its basal two-thirds. Both spinules and alveoli are feebly argentaffin and are stained by the routine stain, the former a very pale pink and the latter red.

The endocuticle is laminated and closely resembles that found in other insects with soft cuticles. Its principal constituent is the normal chitin-protein complex.

II. The Cuticle of Epicoccus sp.

To comprehend the structure of the cuticle of *Epicoccus*, some knowledge of its mode of development is essential.

At the end of a short period of wandering, the second female nymphal instar assumes a permanent position on a twig, petiole, or leaf, moults, and enters her third and final stadium. Rapid development of the cuticle now begins. The cells of the dorsal hypodermis hypertrophy and their secretory activity correspondingly increases. This gives rise to enormous allometric growth of the dorsal cuticle as two lobes, one along either side, whose inner surfaces are in close contact with the bark of the host. Posteriorly these do not clasp the stem but grow round until their margins meet enclosing a small orifice (Fig. 3). While these changes have been in progress, glands located along the parts of the lobes contiguous with the bark have been pouring out large quantities of wax, not unlike bees-wax in colour and texture, which cements the insect firmly to the surface of the stem. Meanwhile, its ventral surface has moved outwards away from the bark, the space so formed constituting a brood chamber (Figs. 2 and 3) whose only means of communication with the exterior is through the pore between the adpressed posterior tips of the lobes. As a result of these changes, the greater part of the cuticle, and the only part visible when the insect is *in situ*, consists of the large thickened dorsum. The remaining much-smaller enclosed region comprises the small thin sternal cuticle and the relatively-minute, degenerate pleural regions. The whole development resembles much more closely that of a coccid than that of a pseudococcid. The thickened dorsum, deeply infolded to expose the least possible area to the atmosphere, the enclosure of the entire thin part of the cuticle, and the spiracles opening into the brood chamber, all appear to be adaptations to minimize water loss.

The fully-developed female (Fig. 4) has an average length of about 3 mm. and is somewhat less than this in width. Transverse sections display a great variety of contour, the only consistent feature being their distorted U-shaped outline. Although an occasional isolated female may be almost bilaterally symmetrical, the normal condition, brought about by the gregarious mode of life, is for one or the

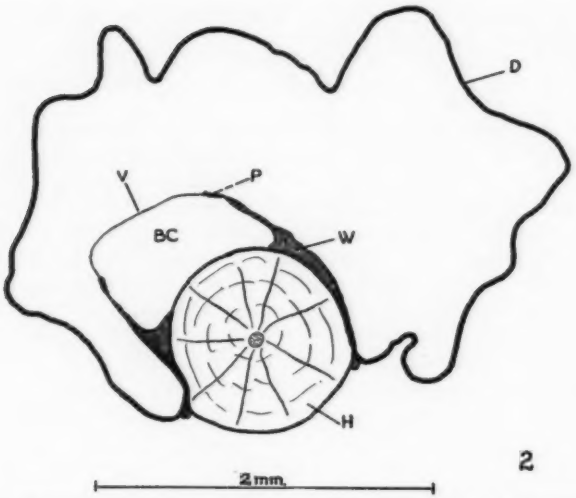

FIGURE 2. *Epicoccus* sp. Transverse section showing spatial relation between insect and host. BC, brood chamber; D, dorsal cuticle; H, host; P, pleural cuticle; V, ventral cuticle; W, cementing wax.

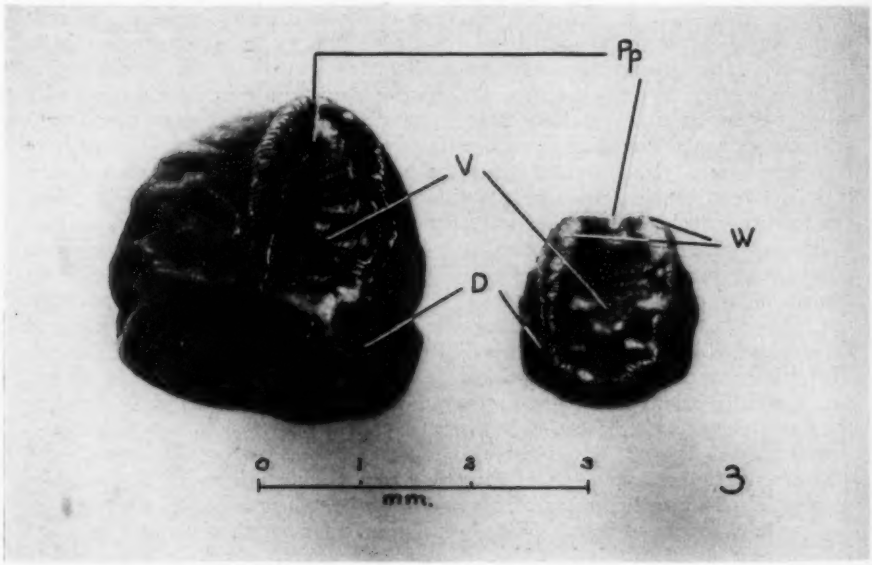

FIGURE 3. *Epicoccus* sp. Fully developed and immature third female instars. Ventral aspect showing brood chamber and posterior pore. D, dorsal cuticle; Pp, posterior pore; V, ventral cuticle; W, cementing wax.

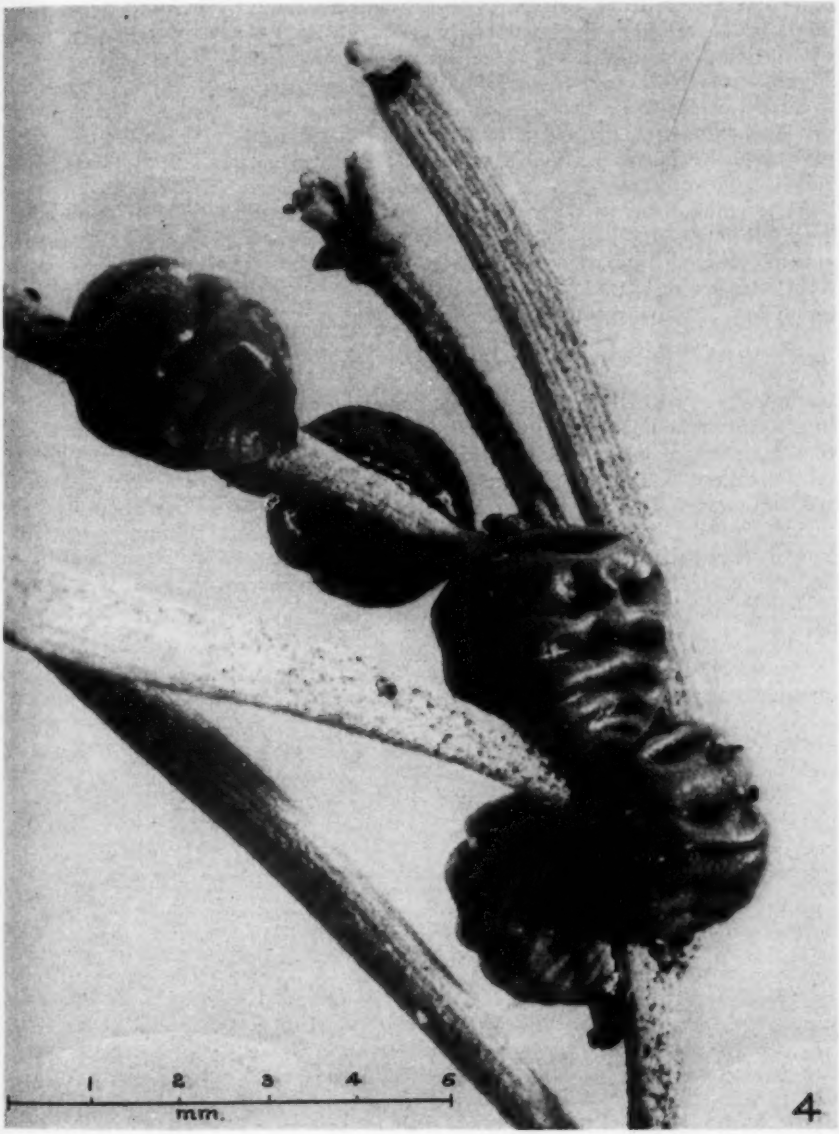

FIGURE 4. *Epicoccus* sp., *in situ*. *Photograph courtesy of Helen M. Brookes.* The females are invariably so oriented that the rounded cephalic end is directed towards the host's region of growth. Part of the cementing wax is visible on the second insect from the top.

other lateral lobe to be more developed than its fellow. Which of these is the larger is determined by the proximity of neighboring females, leaf petioles or similar obstructions, and the natural asperities of the bark encountered during growth. Figure 2 was drawn from a section selected, not because it was typical, but because it clearly displayed the spatial relation between insect and host.

The mouthparts excepted, the only regions of the body capable even of limited movement are the pleura and the ventral abdominal surface, both of which are subordinate to the functions of oviposition and defaecation.

The cuticle exhibits great diversity of thickness, structure and composition. For descriptive purposes, four major types are here recognized: the respective cuticles of the dorsum, the venter, the pleura, and the intersegmental membranes. The latter are restricted to the enclosed ventral surface; externally, they are suppressed by fusion of the segments and are represented only by sutures.

1. *The Dorsal Cuticle*

Relative to the size of the insect, the dorsal cuticle is massive, ranging in thickness from a minimum of 30 μ near its junction with the pleura (Figs. 5 and 6A) to some 40 μ in the remainder (Fig. 6C). Maximum thickness is attained in small localized patches where muscles, particularly those in the abdomen associated with the organs of oviposition, are inserted. In such areas thickenings of 70 μ or more are not uncommon (Fig. 6B). It exhibits a general uniformity of structure throughout, consisting of a two-layered epicuticle overlying the procuticle. It is interesting to note that no part of the cuticle is argentaffin.

A. The Epicuticle

Irrespective of its location, the epicuticle displays a constancy of thickness, structure, and composition so that what is said of it here in connexion with the

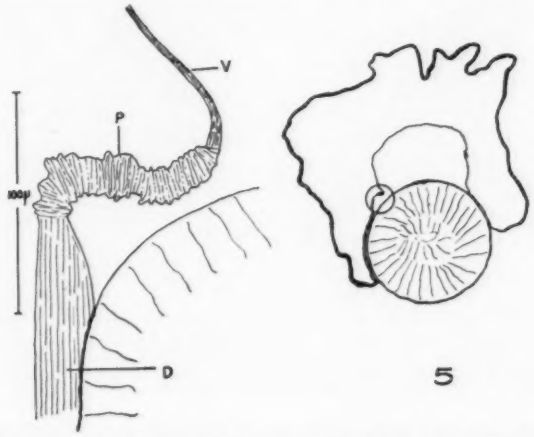

FIGURE 5. *Epicoccus* sp. Dorso-pleural and ventro-pleural junctions. The part of the cuticle drawn is indicated by the small circle in the diagram at right. Symbols as for Figure 2.

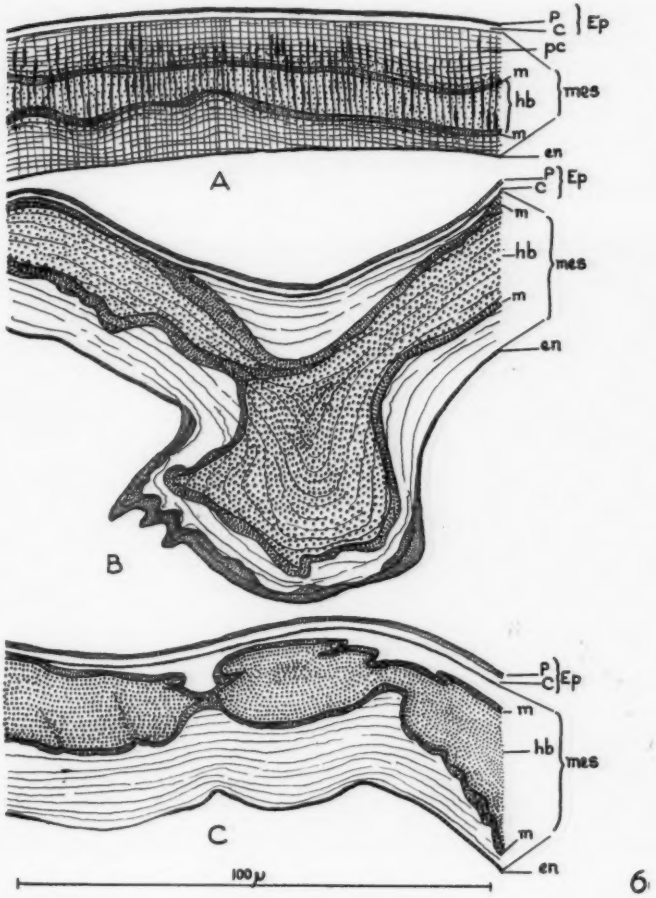

FIGURE 6. *Epicoccus* sp. Dorsal cuticle. A and C, general cuticle; B, cuticular ingrowth. Pore canals are omitted from B and C for clarity. hb, heavily-staining zone; m, margins of heavily-staining zone; mes, mesocuticle. Other symbols as for Figure 1.

dorsal cuticle has general application. Except for the non-pigmented condition of the paraffin layer, it is practically indistinguishable from the same structure in *Pseudococcus*. It has a total thickness of about 1.5 μ distributed between the (outer) paraffin component which constitutes about one third of it, and the (inner) cuticulin layer which comprises the remainder (Figs. 6 and 7).

The Cuticulin Layer:

The cuticulin layer is colorless and transparent. It gives a vigorous response to Millon's reagent, is stained yellow by the xanthoproteic reaction, and orange-

red by the routine stain. It is non-argentaffin, non-reducing, non-iodophil, and is unaffected by Danielli's, Gibb's, or Mallory's PTAH techniques. It gives negative responses to Sudan black B and Nile blue sulfate. It is easily soluble in hot solutions of potassium hydroxide but resists for at least twelve hours the action of 10% hydrochloric acid at 60° C. These responses indicate a composition largely proteinaceous. The materials to which the protein is bound are such that the usual range of tests do not serve for their identification. They also confer on it its resistance to extraction by hot dilute acids.

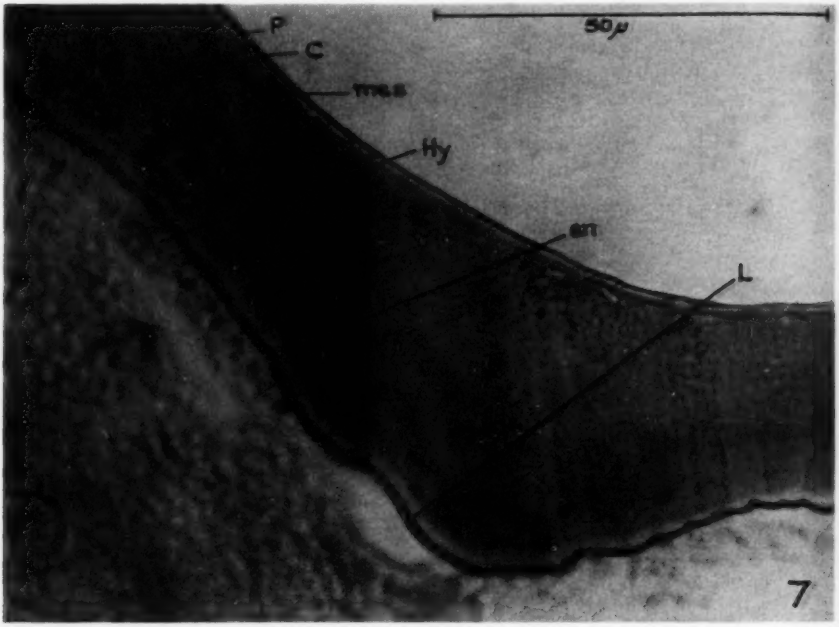

FIGURE 7. *Epicoccus* sp. Dorsal cuticle 4 μ; stained with Sudan black B, bright-field. Hy, hypodermis; L, lipoid zone; mes, mesocuticle. Other symbols as for Figure 1.

The Paraffin Layer:

This layer is colorless. Its characteristic component is lipoid, as the strong positive responses to fat stains (Fig. 7) and osmic acid indicate. Schmorl's test shows it to possess strong reducing properties and Mallory's PTAH stains it deep violet. It is intensely iodophil but it is not affected by the other reagents used nor is it soluble in dilute acids or alkalis.

The Surface Secretion:

The dorsal cuticle of living insects or of those killed with cyanide has a resinous luster (Fig. 4) which is retained when the dead specimens are dehydrated. The

glossy surface cannot be wetted. If freshly killed or dried specimens are treated with fat solvents, 90% ethanol, or warm 5% sodium or potassium hydroxide solution for five minutes, and then washed with water and dried, the luster is lost and the entire surface becomes dull. Treatment with boiling water, hot concentrated hydrochloric acid, or exposure to dry heat at 100° C. for twelve hours does not affect it. Immersion of insects in molten water-soluble wax similarly leaves the luster undimmed.

Untreated and "dulled" insects were therefore embedded in this medium, the sections stained with aqueous dyes in acid solution, and mounted in glycerol which is without effect on the luster. Sections cut from both batches were indistinguishable nor did comparison with sections of fresh material reveal any recognizable differences.

This scanty evidence suggests that a layer of sub-microscopic thickness (possibly containing lipoid) may cover the paraffin layer of the dorsal epicuticle.

B. The Procuticle

The procuticle consists almost entirely of mesocuticle (Fig. 7). Exocuticle is restricted to small widely-dispersed papillae, each bearing a minute hemi-sclerotized seta. The endocuticle forms a narrow zone about 1 μ in thickness except at the termination of cuticular ingrowths where its lobes and thickened portions serve as intermediaries for muscle attachment (Figs. 6 and 7).

The Exocuticle:

In the sense that exocuticle is completely-sclerotized procuticle, this zone is wanting in *Epicoccus*. Both papillae and setules are transparent; the former are very pale yellow, the latter are colourless. Neither is inert to aniline stains as is true exocuticle. The routine stain colours both of them pink and the colour deepens as time of immersion is increased. Treatment for a few minutes with "Diaphanol" is sufficient to destroy the incipient sclerotization and they then stain intensely and rapidly with acid dyes. Their development appears to be in a stage intermediate between mesocuticle and exocuticle. Complete sclerotization commonly induces changes in the overlying cuticulin layer but the epicuticle of the papillae differs in no respect from that of other parts of the body.

The Mesocuticle:

The mesocuticle shows little structural differentiation. When unstained sections are examined under the highest powers of the light microscope the mesocuticle appears uniform and featureless. Under phase-contrast conditions it is transversely marked with numerous, irregular dark streaks which indicate the positions of pore canals of whose organization, however, no details are observable. The routine stain dyes the zone red but supplies little information on canal structure.

Chemically, the mesocuticle is as complex as it is structurally simple. Millon's reagent differentiates it into three clearly-defined sub-zones which, beginning with the outermost, are here referred to as the A, B, and C sub-zones, respectively (Fig. 8).

The A sub-zone is stained pink by Millon's reagent, and the distal parts of the pore canals appear as indistinct, thin red lines which, after traversing the region,

attenuate before they terminate at its outer surface. It is of variable width; in some parts it may form as much as a third of the cuticle but is generally less. Occasionally it is suppressed by the outward extension of the B sub-zone (Fig. 6B).

The B sub-zone takes the form of a conspicuous, broad, bright-red band, sharply demarcated externally and internally from the remainder of the procuticle by narrow crimson margins (Figs. 6 and 8). It is continuous throughout the dorsal mesocuticle, though it thins before it terminates at the dorso-pleural junctions. This intensely-staining proteinaceous region exhibits great and sudden variations in thickness. Its maximum development may be observed in cuticular ingrowths of which it occupies the greater part (Fig. 6B). Where it is thinnest (Fig. 6C), it is composed solely of the contiguous crimson margins. The distinct differences

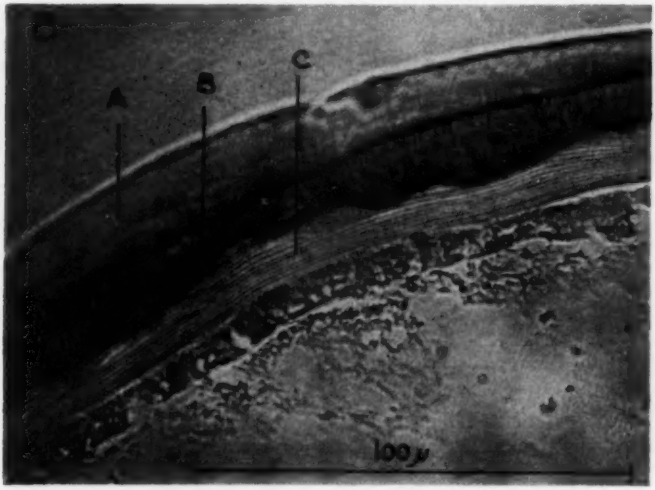

FIGURE 8. *Epicoccus* sp. Dorsal cuticle, 4 μ. Millon's reagent; bright field. A, B, and C are the three sub-zones referred to in text. Maximum definition in the mesocuticle has been sought; the epicuticle is distorted through being out of focus.

in color between the central part and its marginal bands suggest either that different proteins are present in each or that the concentration of the one protein is higher in the margins than internally. The general appearance is reminiscent of the result obtained by the use of the paper-chromatography technique. The great mass of the pore canals (Fig. 9) is located within the zone and it is possible that a protein complex in solution diffuses from them into the circumjacent cuticle there to undergo partial separation, the chitinous matrix acting similarly to the chromatographic paper.

Sub-zone C is stratified in alternate red and pale-pink layers (Fig. 8). Whether or not these coincide with the probable original lamellate deposition of the procuticle cannot be determined since no other stain or technique used, produced comparable differentiation. This sub-zone varies in thickness but occupies about the inner third of the mesocuticle.

Of the wide range of tests applied to the mesocuticle none so clearly distinguished the sub-zones as did Millon's reagent. Other tests which responded positively gave diffuse results, but confirmed the fact that the concentration of protein in the B sub-zone is higher than in any other part of the mesocuticle. Ninhydrin colored the mesocuticle violet-pink, the greatest depth of color being developed in the B sub-zone. The xanthoproteic reaction stained it deep orange medially, paling to yellow towards either surface. The iodine technique (Lower, 1957b) approximately delineated the sub-zone by staining it deep purple-black, the C sub-zone was uniformly dark red, and the A sub-zone was practically unstained. Mallory's PTAH produced a similar picture, the B sub-zone being reddish violet with deeper violet margins, the C sub-zone light red, and the A sub-zone almost colorless.

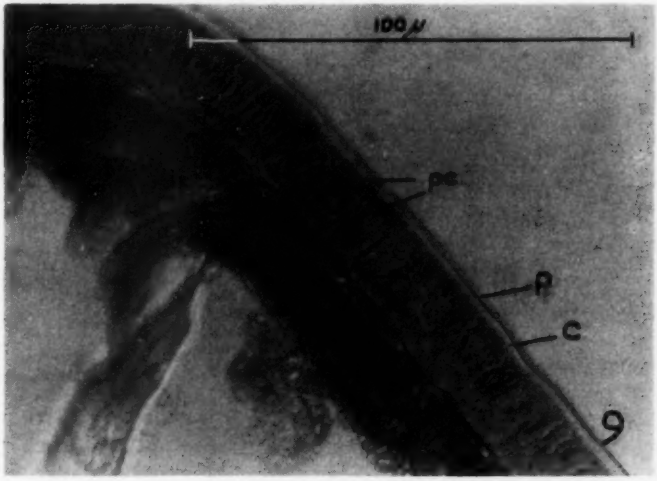

FIGURE 9. *Epicoccus* sp. Pore canals in latero-dorsal cuticle. One micron; stained by Sevki's technique; bright-field. Note termination of pore canals at outer mesocuticular surface. C, cuticulin layer; P, paraffin layer; pc, pore canals.

As is usually the case, Sevki's technique produced the clearest differentiation of the pore canals (Figs. 9, 10). Their thin hypodermal connections were colored red, their thickened portions in the B sub-zone were deep purple, and their thin terminal parts in the A sub-zone were red. Their numerosity is such that even in sections cut at 1 μ they appear as a confused mass (Fig. 10). The only valid conclusions that can be drawn concerning them are the following: the pore canals are confined to the procuticle, being continuous between its outer surface and the hypodermis; they are extremely numerous, of highly irregular form, and the mass of their contents, which possess a high concentration of tyrosine-containing protein, is almost entirely located within the B sub-zone.

The mesocuticular protein is separable into two fractions. Sections were immersed for twelve hours in 10% hydrochloric acid maintained at 60° C. After

washing in distilled water it was found that the mesocuticle had lost its characteristic staining properties. Millon's reagent colored it uniformly pink and the xanthroproteic reaction pale yellow. Ninhydrin, Mallory's PTAH, and the Sevki and iodine techniques all gave negative results. The routine stain dyed it light red. In no section were pore canals visible and even the use of phase-contrast failed to reveal their positions. These results demonstrate that while most of the protein is extractable with hot dilute hydrochloric acid, there is a residual fraction firmly bound to the chitin in such a manner that its extraction is more difficult.

There is no evidence to suggest either the mode of accumulation or functioning of the apparently high protein concentration in the B sub-zone. The adult male of either species of *Epicoccus* is unknown; the cuticles of first and second nymphal

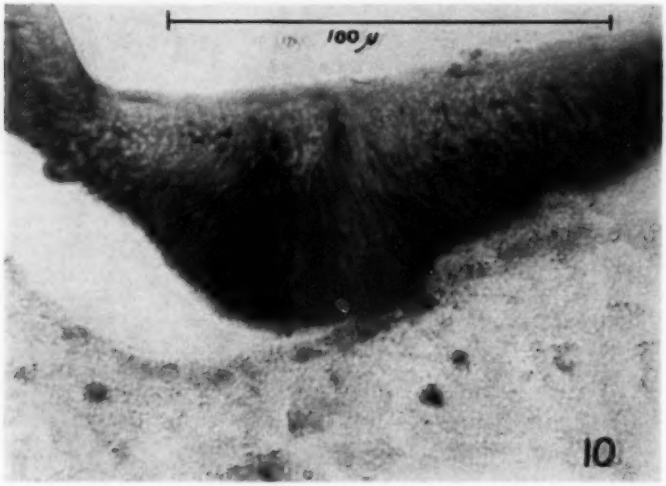

FIGURE 10. *Epicoccus* sp. Pore canals in thick part of dorsal mesocuticle. One micron; stained by Sevki's technique; bright field.

instars of either sex do not differ significantly from those of the corresponding instars of other pseudococcids. Of the known stages, therefore, the phenomenon is confined to the third female instar in which the thickness of the sub-zone relative to that of the cuticle as a whole increases with the age of the insect.

The Endocuticle:

As mentioned above, the endocuticle is greatly reduced. It is wanting in many parts of the cuticle, and, where present, is never more than 1 μ in thickness except where it caps the cuticular ingrowths. Its structure appears normal.

The Lipoid Zone:

When the routine stain is applied to a cuticular section, a thin layer, staining a much deeper green than does the endocuticle, can be seen to separate the latter

from the hypodermal cells. This layer has an average thickness dorsally of about 1 μ, and is continuous throughout the dorsal cuticle except where interrupted by cuticular ingrowths. Laterally, it attenuates until it disappears in those parts of the dorsal lobes which are in contact with the bark. It is absent from the cuticles of the pleura, venter, and intersegmental membranes. In unstained sections, the layer is indistinguishable from the endocuticle, since the structure and natural color of both are the same. Its high content of chitin shows it to be definitely of procuticular origin, and but for its impregnation with other materials, it would merely represent the innermost part of the endocuticle.

It is remarkable for its high lipoid content, and to obviate additions to the terminology, unjustifiable at this stage, it is referred to descriptively as the *lipoid zone*. The lipoid zone has unusual chemical properties for a part of the procuticle, so anatomically located. In addition to the reactions characteristic of endocuticle generally, it is stained deep violet by Sevki's technique and Mallory's PTAH. It is most intensely colored, however, by the fat stains.

In free-hand sections of fresh material, the zone can be deeply stained at room temperature by short immersion in the fat stains. Sections cut from paraffin-embedded material, or those cut from cuticles which have been repeatedly extracted with boiling cyclohexane, cannot be stained in this manner. Nearly as intense color, however, can be developed by immersion for several hours at 60° C. in the same stains. The Nile blue sulfate technique shows that part, at least, of the lipoid is neutral, since the zone is stained red after differentiation. If sections cut from extracted or paraffin-embedded material be gently warmed with fuming nitric acid, the chitin-lipoid association is destroyed, and the previously-dispersed lipoid aggregates into minute droplets which stain rapidly and intensely with fat stains. These results indicate that a lipoid complex, rather than a single lipoid, is involved. Part of the lipoid is free, and easily extractable; part is bound to the chitin and the other constituents of the procuticle of the region, and resists extraction.

Of the origin and function of the lipoid zone, nothing is known. Examination of females in various developmental stages shows that lipoid impregnation synchronizes with secretion of the procuticle, and that the zone maintains its position relative to the hypodermis throughout the development. It is not present in the first and second nymphal instars, and was absent from one very young third female instar which had reached this stage only about the time that the insects were collected. In all the other females examined, no measurable difference in its thickness was observed, and in two exceptionally large specimens (both parasitized), the zone was of normal thickness.

Only twice previously has the presence of a layer between the endocuticle and the hypodermis been recorded in the literature. The two records are those of Schmidt (1956) and Malek (1956).

Schmidt reported that, in certain insects which he had examined, a glycoprotein layer, the "sub-cuticle," separated the endocuticle from the hypodermis. As he stated categorically that the "sub-cuticle" was non-chitinous, it clearly has no affinity with the lipoid zone of *Epicoccus,* and need not be further discussed here.

Malek demonstrated that when the desert locust, *Schistocerca gregaria,* is moulting, the inner part of what was originally endocuticle becomes impregnated with a lipo-protein complex, to form the ecdysial membrane of the insect.

This membrane and the lipoid zone of *Epicoccus* appear to be homologous struc-

tures. Since both are derived from the innermost part of the procuticle contiguous with the hypodermis, both occupy corresponding anatomical positions. Both are impregnated with lipoid, and both, by reason of their derivation, contain chitin.

The stages of the life cycle in which each is present are, however, completely reversed. In *Schistocerca*, the lipoid-impregnated procuticle must, by forming an ecdysial membrane, be necessarily confined to immature stages, since the adult does not moult. The lipoid zone of *Epicoccus*, on the contrary, is found in the adult only. Few details are given in Malek's preliminary note, and it will be necessary to await his full account, before an adequate comparison of the two structures can be made.

2. *The Ventral Cuticle*

The cuticle of the venter is thin, averaging some 4 μ in thickness. Its epicuticle is indistinguishable from that of other parts of the body.

A. The Procuticle

There is no exocuticle. Less than half of the procuticle consists of mesocuticle which displays no signs of the chemical sub-zonation characteristic of the dorsal mesocuticle. It has no visible internal structure and chemically, is typical of this zone generally.

The endocuticle comprises rather more than half of the procuticle, being thicker than that of the dorsum. No lipoid zone is present. Both chemically and structurally it resembles the endocuticle of *P. adonidum*.

3. *The Pleural Cuticle*

What is here assumed to be the cuticle of the degenerate pleura covers two narrow regions, one on either side, which connect the ventral and dorsal cuticles. From both of these the pleural cuticle differs greatly in structure. In transverse sections, its surfaces are irregular (Fig. 5) and its general appearance suggests that it is in a contracted state; it is possible that in the living insect it may be much more extended and correspondingly thinner.

It is frequently thick, exceeding 30 μ in a few places. The epicuticle is typical. The procuticle consists wholly of endocuticle which, when stained, shows no structure under bright-field conditions. When viewed under phase-contrast conditions, whether stained or not, numerous, fine, approximately-transverse dark lines can be seen. These appear to be artifacts produced by the contraction assumed to have occurred. The change from pleural to dorsal, or pleural to ventral, cuticles is sharp; there is no gradation of one region into the other.

Tests reveal the presence of the normal chitin-protein complex; there are no unusual components.

4. *The Cuticle of the Intersegmental Membranes*

Functional intersegmental membranes are found uniting the ventral abdominal segments only, so that cuticle of this kind is restricted to the ventral surface.

It is very thin and consists of the typical epicuticle overlying a procuticle rarely

exceeding 2 μ in thickness and often being less. Its highly plicate condition in many places shows that it permits of considerable movement of the ventral region. The procuticle, which is composed wholly of endocuticle, has no visible internal structure, and no peculiar chemical properties. There appear to be no pore canals in it.

III. The Cuticle of E. acaciae (Maskell)

The cuticle of *E. acaciae* differs in details, only, from that of its congener. The species is somewhat smaller than is *Epicoccus* sp. but relative to the size of the insect its cuticle is still massive.

The epicuticles of both forms are indistinguishable even to the extent that dorsally the paraffin layer of each is covered by a sub-microscopic surface secretion of similar properties.

What has been said of the cuticles of the venter, the pleura, and the intersegmental membranes of *Epicoccus* sp., applies equally to those of the same regions of *E. acaciae*, such differences as do occur being confined to the dorsal procuticle.

The endocuticle of the latter has undergone still further reduction and forms irregular cappings to terminations of the cuticular ingrowths. It does not occur elsewhere. The procuticle thus consists almost entirely of mesocuticle which structurally resembles that of *Epicoccus* sp.; its distinguishing characters are chemical. Millon's reagent colors it uniformly cherry-red—a much deeper shade than is produced by the reagent in insect cuticle generally. The xanthoproteic test stains it uniformly deep orange, the iodine technique purple-black, ninhydrin violet-pink, and Mallory's PTAH deep purple. Sevki's technique displays the pore canals as deep violet, filamentous tubes of approximately constant diameter throughout their length and demonstrates their continuity between the hypodermis and the outer procuticular surface. After twelve hours' extraction at 60° C. with 10% hydrochloric acid, Millon's reagent stains the whole procuticle pink, and the xanthoproteic test colors it yellow. It fails to give visible responses with the other stains and reagents.

These results would seem to indicate that extra protein is present in the mesocuticle, that it is uniformly distributed (in contrast to its aggregated condition in *Epicoccus* sp.), and that it is differentiated into acid-extractable and acid-resistant fractions.

A lipoid zone, whose extent and reactions are identical with those of the corresponding zone of *Epicoccus* sp., is present. It differs in being brown pigmented with melanin or a melanin-like product, and is hence easily recognizable even in unstained sections.

IV. The Wax Glands and Their Secretion

(a) *P. adonidum*

Wax glands are conspicuous in most sections of *P. adonidum*, frequently as many as four or five, transected in various places, being visible in the one section. They resemble those of *P. martimus* as described by Pollister (1937) and it is probable that a general uniformity of glandular structure prevails throughout the family. Fundamentally, each consists of a multicellular secretory sheath enclosing a large,

sub-spherical, central reservoir which communicates with the surface by means of one or more ducts (Fig. 1A).

When sections of unfixed material are treated with Sudan black B, the surface wax, the contents of reservoirs and ducts, and limited parts of the cuticle surrounding the ducts, are blackened. The local cuticular impregnation appears to be brought about by diffusion of some of the wax through the duct walls. The Nile blue sulfate technique stains the surface wax, that filling the ducts, and the impregnated cuticle deep blue, but it colors the reservoir contents red. Fat solvents readily dissolve the surface wax and that of the ducts but have no apparent effect on the impregnated cuticle or the reservoir material. The latter is highly resistant to such solvents; prolonged extraction with methanol-chloroform, pyridine, ether, or boiling cyclohexane removes part only of its lipoid. This explains why even in paraffin sections cut at 1 μ the contents of the reservoirs are retained apparently unaltered by the treatment they have undergone in the course of their preparation.

These results suggest that the material in the reservoir is "protowax" consisting of neutral lipoid (indicated by the Nile blue sulfate technique) so bound to other substances as to render its extraction extremely difficult. Alternatively the protowax may be secreted as an emulsoid whose finely divided lipoid micelles are dispersed in an inert medium. By some means at present unknown, the lipoid is freed and passed along the ducts. On its way to the surface the neutral lipoid becomes acidic, in which form it is deposited as wax on the surface. This interpretation of the course of events is purely speculative. An important obstacle to its acceptance is the failure of any of the wide range of tests applied, to demonstrate the presence in the reservoir of anything except lipoid.

There is some evidence suggesting that the outer surface of the wax layer may be covered by a sub-microscopic layer of protective material.

(a) If insects are killed with cyanide and then immersed in a solution of Sudan black B, the surface wax is stained only where it has been damaged during manipulation. If they are first lightly brushed, the areas so treated stain rapidly.

(b) If the insects have a prior immersion for fifteen minutes in 10% hydrochloric acid at 35° C. before being put in Sudan black B, staining of the wax is rapid and complete. Moreover, from insects so pre-treated, cold cyclohexane dissolves the wax rapidly whereas it acts much more slowly on untreated insects.

(c) If the insects are dropped into water at 70° C., the wax oozes away forming a surface film on the water. If such insects are then embedded in paraffin and sectioned, the epicuticle, when viewed under phase-contrast conditions, has an outer surface which cannot be sharply focussed. If the same sections are then treated with warm 10% hydrochloric acid for ten minutes, and re-examined, the epicuticle has a well-defined outer boundary.

Should an outer layer be present, the wax and its protective layer would afford an interesting analogy to the two outer layers of the four-layered type of epicuticle. They would occupy the same anatomical position relative to the two inner layers, and apparently perform the same function of limiting water loss as do the wax and cement layers. They differ in that the wax at least is a glandular product, they are not secreted until after moulting, and finally, some of the wax is used by many species as a covering for egg masses.

(b) *Epicoccus*

In *Epicoccus* most of the wax glands have atrophied. Their orifices are still open, but either their ducts are internally sealed off by the growth of procuticle across them, or their secretory cells are small and produce little wax. Glands of the latter kind are confined to the dorsum where they open in small groups in deep infolds of the cuticle adjoining muscle insertions. They secrete little more wax than is needed to plug the ducts and keep them filled. Their orifices are marked by small white spots at the surface (Fig. 4).

Typical glands are confined to the lateral extremities of the lobes in contact with the bark. The gland contents and the cementing wax respond similarly to stains and tests as do those of *P. adonidum*. The principal differences between the waxes of the two species are that that of *Epicoccus* has a higher melting point, and dissolves easily and quickly in all fat solvents. It would appear that the wax having ceased to function as an agent for reducing desiccation, no protective layer covers it.

V. Discussion

The results of this investigation demonstrate that notwithstanding the fundamental similarity of cuticular structure which prevails throughout the Pseudococcidae, great morphological differences distinguish the two genera studied.

A. The Epicuticle

The epicuticles of all three species consist of the essential cuticulin and paraffin layers. The paraffin layer of *Epicoccus* is overlain by a sub-microscopic covering of apparently lipoidal material which probably corresponds to the wax layer of more complex epicuticles, as a cement layer in this anatomical position would be abnormal. There is no indication of the presence of any such layer in *Pseudococcus* in which it is replaced by a thick layer of wax secreted by hypodermal glands; this surface wax itself appears to possess an extremely tenuous protective covering.

The effectiveness of either of these systems as a means for restricting water loss is probably slight. Much of the surface wax of *Pseudococcus*, for example, is disposed in long filaments which would have little value in this regard, more especially for a species which inhabits a humid micro-environment. Dead insects of either genus, after removal of the surface layer, do not lose water to a dry atmosphere at a significantly greater rate than do dead intact ones.

B. The Procuticle

The procuticles of the two genera differ as greatly as do the environments inhabited by each. The thin procuticle of *Pseudococcus*, consisting almost solely of endocuticle, displays little specialization. That of *Epicoccus*, on the contrary, is highly specialized both structurally and chemically.

In absolute thickness it is comparable with those of large sclerotized forms such as *Periplaneta*, while relative to the individual's size, there are probably few other insects which can match it. It consists almost wholly of mesocuticle.

Among insects whose cuticles have been described, that of *Epicoccus* is unique in the high proportion of protein in the procuticle, aggregated in a well-defined zone.

Its possession of a deep-seated lipoid zone in the procuticle is, so far as is known, shared only by the desert locust, *Schistocerca gregaria*.

All this specialization betokens a long period of evolution under conditions adverse to insect life generally, and the acquisition of a massive cuticle, together with the loss of mobility by the adult female, are presumably closely linked with this. Incapacity for locomotion may be disadvantageous; but failure to cope with a hostile environment spells extinction.

The collected material came from a single half-dead plant, not over ten feet in height. Fully-exposed as the insects were, they not infrequently had to contend with direct sun temperatures of 60° C., or even more, accompanied by relative humidities often below 10%, in a situation commonly swept by parching winds. The brood chambers of mature females contained eggs, and first and second nymphal instars. These immature forms were found nowhere else. Apart, therefore, from ensuring survival of the mother, the thick leathery cuticle functions equally well in protecting those stages in the life cycle most vulnerable to the environmental conditions, thereby making it possible for the species to maintain itself in a region relatively-poor in the higher forms of insect life.

I wish to thank Miss Helen M. Brookes of this department for supplying the material used and for allowing publication of the photograph reproduced in Figure 4.

My thanks are also due to Mr. Keith P. Phillips (in charge of Photographic Department) who is responsible for all the photography.

Summary

1. The cuticular structure of three female pseudococcids, *Pseudococcus adonidum* L., *Epicoccus* sp., and *E. acaciae* (Maskell), has been investigated.

2. The cuticle of *P. adonidum* consists of a two-layered epicuticle, overlying a thin procuticle, almost all of which is endocuticle.

3. The cuticle of *Epicoccus* sp., is highly specialized. Its epicuticle closely resembles that of *P. adonidum*. The dorsal cuticle is relatively thick, and is much modified chemically. Most of it consists of mesocuticle in which Millon's reagent delimits three well-defined zones which differ greatly in their reactions to stains and histochemical reagents. The endocuticle is much reduced. A thin layer of procuticle between the hypodermis and the endocuticle is impregnated with lipoid to from a "lipoid zone."

4. The cuticle of *E. acaciae* is thick. It differs from that of *Epicoccus* sp. principally in that there is no chemical zonation of the procuticle, and the lipoid zone is melanin-pigmented.

5. Wax glands are numerous in the cuticle of *P. adonidum*. The contents of their reservoirs ("protowax") differ chemically from the surface wax. In *Epicoccus*, many of the glands have atrophied; typical glands are confined to lateral parts of the cuticle in contact with the host plant, and these secrete large quantities of wax which fixes the insect permanently in position.

6. The specialized cuticle of *Epicoccus* appears to have evolved over a long period, during which the insects have been exposed to adverse environmental conditions.

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PHENYLTHIOUREA TREATMENT AND BINDING OF RADIOACTIVE IODINE IN THE TADPOLE

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In a study of the distribution of I^{131} after administration to tadpoles, Dent and Hunt (1952) demonstrated not only the expected concentration of this substance in the thyroid gland but also significant accumulations in several other regions. Notable among these were the thymus, the horny teeth, the melanophores of the skin, and the pigmented layer of the retina. The same pattern of distribution was observed in tadpoles of *Hyla, Rana,* and *Bufo* in various stages of development and was not altered by thyroidectomy. It was suggested that, since tyrosine is a precursor of melanin, the localization of I^{131} in pigmented tissues may be attributable to the binding of I^{131} to tyrosine that must be present in those tissues. The hypothesis was also advanced that perhaps the same enzymes that bring about oxidation of tyrosine to melanin are able to facilitate the union of iodine and tyrosine. Gennaro and Clements (1956a, 1956b), studying the binding of I^{131} in the skin of the adult frog, provided evidence to support these views. Our experiments were undertaken as a further investigation of the association of iodine with pigmented tissues and as a further test of the hypothesis cited.

It is known that administration of certain derivatives of thiourea results in an inhibition of melanin formation. This has been demonstrated for mammals (Richter and Clisby, 1941; Dieke, 1947), fishes (Frieders, 1954), and several different amphibians (Lynn and de Marie, 1946; Lynn, 1948; Blackstad, 1949; Millott and Lynn, 1954). In amphibians the results are most striking when these substances are given to embryos or young larvae before any melanophores have appeared. Such individuals do not develop black pigment so long as the treatment is continued. Cessation of treatment is followed by rapid melanogenesis. Since it has been demonstrated (Bernheim and Bernheim, 1942; Paschkis, Cantarow, Hart and Rakoff, 1944; Dubois and Erway, 1946) that thiourea derivatives inhibit tyrosinase activity *in vitro,* it is assumed that their role in preventing melanin formation in frog embryos is the inhibition of tryosinase activity. In our experiments, larvae of several different ages were treated with one of these tyrosinase inhibitors, phenylthiourea, to obtain unpigmented tadpoles or tadpoles with reduced pigmentation. The pattern of iodine uptake in these animals was compared with that in untreated controls at various times after the beginning of treatment.

Materials and Methods

The animals used for this experiment were tadpoles of *Hyla versicolor versicolor* LeConte hatched from eggs collected in a small temporary pool near Oak Ridge,

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Tennessee. A few hours after hatching, the larvae were distributed in groups of fifteen in finger bowls, each containing 200 ml. of spring water. On the first, fourth, and fifteenth days after hatching, experimental series were established in this way: Larvae were transferred to six finger bowls, fifteen larvae to each bowl; two contained 200 ml. of 0.01% phenylthiourea in spring water, two contained 200 ml. of 0.005% phenylthiourea in spring water, and two contained spring water alone (controls). The tadpoles, kept at laboratory temperatures (21°–23° C.), were fed crumbled pellets of Purina rat chow and, occasionally, a strained beef-and-liver soup prepared for infants. The culture fluids were changed daily and there was no mortality. At four-day intervals three animals from each experimental and control group were selected at random and examined under the binocular microscope. Records were kept of the gross changes in pigmentation and of the developmental stage reached. The system of staging devised by Taylor and Kollros (1946) for *Rana pipiens* was used and adapted with minor variations for *Hyla versicolor*.

At three ages (4, 24, and 29 days after hatching), animals were removed from the experimental and control series and used in the preparation of autoradiograms.[4] The procedure was as follows: Five larvae from each bowl were put in 50 ml. of a solution of one part per million of stable sodium iodide [5] and enough radioiodine to give an activity of one μc./ml. at the beginning of the immersion period. For each experimental group, one set of five animals was put in a radioiodine solution made up in spring water and another was put in a radioiodine solution made up in the same phenylthiourea solution in which the animals had been raised. After 24 hours in the radioiodine solution, the larvae were passed through two baths of spring water and left in a third bath of spring water, or the appropriate phenylthiourea solution, for another 24 hours. All were then fixed in a 1:1 mixture of Bouin's fluid and Cellosolve. After 8 hours' fixation, they were dehydrated in Cellosolve, embedded in paraffin, and sectioned at 10 micra. The mounted and dried sections were passed through two changes of xylol, transferred to absolute alcohol, and then dipped in a 1.0% solution of collodion in ether-alcohol, and dried. The slides were attached by stationer's binder clips against the emulsion of Eastman medium contrast lantern slide plates in the darkroom and left for 8 days. Finally, the lantern slide plates were developed and the sections themselves were stained with Harris' haematoxylin and Ponceau de xylidine-orange II (Gray, 1952).

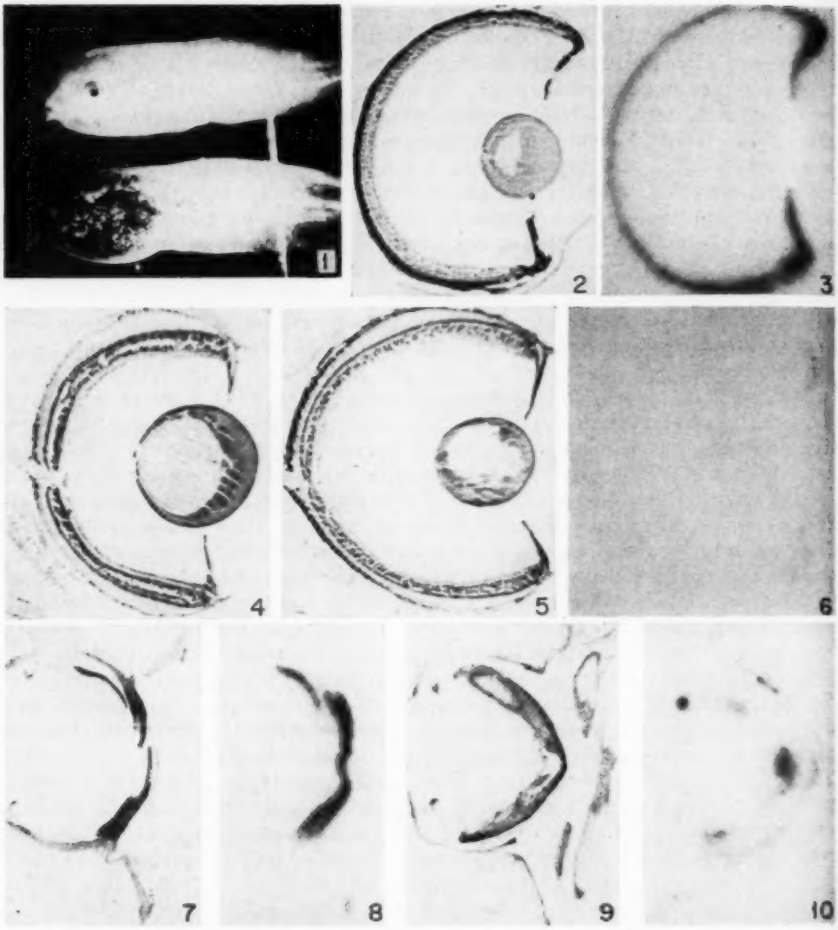
Results

1. *Gross effects of treatment with phenylthiourea*

During ovogenesis (Kemp, 1953) melanin granules are laid down in the cortical region of most anuran ova. Superficially, the pigmented area extends from the animal pole to the presumptive germ ring. These granules are retained within

[4] Joftes and Warren (1956) have recommended the substitution of the term "radioautogram" for "autoradiogram." It is felt that since the semantic basis offered by Joftes and Warren for the use of the former term does not appear to be much stronger than the etymological basis presented by Boyd (1955) for the use of the latter and since the latter term has become well established, its use should be continued.

[5] There is some danger of introducing errors by use of carrier-free isotopes because of their tendency to adhere to glassware. The stable NaI was added with the view of eliminating that effect.



All sections shown are from 26-day-old larvae of *Hyla versicolor* that had been immersed in a solution of one μc of I^{131}/ml. for 24 hours and fixed 24 hours after removal from the solution.

FIGURE 1. Photograph of living tadpoles 12 days old. Upper animal raised in 0.01% phenylthiourea; lower animal raised in spring water.

FIGURE 2. Section through the eye of a control tadpole.

FIGURE 3. Autoradiogram prepared from the section shown in Figure 2.

FIGURE 4. Section through the eye of a tadpole kept in 0.01% phenylthiourea continuously.

FIGURE 5. Section through the eye of a tadpole kept in 0.01% phenylthiourea for 24 days, then in spring water for two days.

FIGURE 6. Autoradiogram of the section shown in Figure 5.

FIGURE 7. Section through the mouth of a control tadpole showing the horny teeth.

FIGURE 8. Autoradiogram of the section shown in Figure 7.

the presumptive ectoderm and mesoderm but disappear as melanophores begin to differentiate and form melanin.

As was anticipated on the basis of previous experiments with other amphibians, the larvae that had been put in phenylthiourea solutions within a few hours after hatching stopped forming melanin and after only 24 hours were noticeably paler than controls. After 48 hours, and at all later stages, the experimental animals were completely unpigmented, the embryonic pigmentation having disappeared (Fig. 1). Larvae in which treatment was delayed until four days after hatching had already developed much melanin in both skin and eyes and exhibited no difference from the controls for at least three days. After this they gradually paled, however, and by the tenth day of treatment their skin was without pigment. Pigmentation of the eyes was lost much more slowly and even when the experiment was terminated (51 days after hatching), the eyes of these animals still showed some pigment, though far less than those of controls. Larvae that were first put into phenylthiourea solutions 15 days after hatching showed no blanching of the skin until near the end of the experiment, and the pigmentation of the eyes never became grossly different from that of controls. Examination of the living animals under the binocular microscope and later study of the sectioned material revealed no significant pigmentary difference between animals treated with 0.01% phenylthiourea and those treated with 0.005%. It appears that both concentrations are fully effective in inhibiting melanin formation.

Since phenylthiourea is one of the well-known thyroid-inhibiting drugs, the experimental animals not only differed from the controls in degree of pigmentation but also in their failure to exhibit definitive metamorphic changes. No significant differences in development were noted for the first 20 days of the experiment. At this time both treated and untreated tadpoles were in late limb bud stages (Stages IV and V). Later, however, the experimental animals showed definite inhibition in development. By 24 days the controls were in Stages VI and VII whereas the tadpoles in phenylthiourea solutions remained at Stages IV and V. The controls continued to differentiate steadily, most specimens reaching Stage IX by the 28th day, Stage XV by the 40th day, and late metamorphic stages (Stages XVIII to XXV) by the 48th day. As is usual with anuran larvae, there was a considerable variation in developmental rate among the controls, a few specimens being retarded and others exceptionally advanced. Thus, although forelimb emergence occurred in one control 45 days after hatching, and had occurred in more than half the surviving controls by 51 days, at this latter time there was still one animal at Stage VI and several at Stage XIII. Among the tadpoles placed in 0.01% phenylthiourea, either immediately after hatching or 4 or 15 days later, none advanced beyond Stage VI and most remained at Stage IV or Stage V. Tadpoles raised in 0.005% phenylthiourea advanced somewhat beyond those in the higher concentration; most of those kept to the end of the experiment reached Stage VI or Stage VII and a few specimens differentiated to Stage VIII. There is thus some indication that the lower concentration is not fully effective in inhibiting thyroid activity.

FIGURE 9. Section through the mouth of a tadpole treated continuously with 0.01% phenylthiourea.

FIGURE 10. Autoradiogram of the section shown in Figure 9.

As noted previously, the larvae used for the preparation of autoradiograms were put in a radioiodine solution for 24 hours, and this was followed by another 24-hour period without radioiodine, which allowed for elimination of excess iodine before fixation of the tadpoles. Autoradiograms were made from two sets of phenylthiourea-treated animals, one in which the phenylthiourea treatment was continued until fixation and one in which the radioiodine solution and the subsequent 24-hour bath were without phenylthiourea. The latter thus had a 48-hour period of recovery from phenylthiourea treatment immediately preceding fixation. It has been demonstrated that in amphibian embryos melanin reappears very rapidly after phenylthiourea treatment has stopped (Millott and Lynn, 1954). In the present experiments the animals put in radioiodine solutions containing no phenylthiourea showed a well-defined darkening of the eyes within 12 hours; and at 24 hours, when they were removed from the radioiodine solutions, a scattering of pigmented melanophores was also visible in the skin. Larvae kept in phenylthiourea solutions throughout, of course, showed no such pigmentary change.

2. Effects of phenylthiourea treatment on radioiodine binding in the pigmented epithelium of the retina

The first group tested for radioiodine binding consisted of animals on which the phenylthiourea treatment started on the day of hatching and continued for four days only. The second and third groups tested included animals on which treatment was begun on the day of hatching and some in which treatment was started later (4 and 15 days after hatching). Since the results were similar in all three groups, detailed consideration will be given for only one, the second experimental group, which was given radioiodine on the 24th day after hatching and contained animals under treatment for 24, 20, and 9 days.

Photomicrographs and corresponding autoradiograms of the eye region in typical animals of this group are shown in Figures 2–6. Figures 2 and 4 illustrate the conditions found in control and experimental animals of the series in which phenylthiourea treatment was started at hatching. The pigmented epithelium of the retina is quite dark in the controls but is entirely without melanin in the animals given continuous phenylthiourea treatment. The autoradiogram prepared from the control (Fig. 3) shows that there was a marked concentration of radioiodine in the pigment epithelium. On the other hand, an autoradiogram prepared from the eye shown in Figure 4 was entirely blank and is not shown. It has been pointed out that some melanin formed in the animals allowed a recovery period from treatment with phenylthiourea. Plates prepared from these specimens show faint but definite autoradiograms, indicating that some binding of radioiodine occurred both in the skin and in the retina (Figs. 5, 6). On the basis of these results it might be concluded that the binding of radioiodine depends on the presence of melanin and varies directly in amount with the amount of melanin present. However, study of the larvae in which phenylthiourea treatment was initiated at later ages reveals that this is not the case. It will be remembered that pigment was lost from the eyes only very slowly in the series started at 4 days and not at all, so far as could be seen externally, in the series started at 15 days. Thus these latter tadpoles, even though under continuous treatment with phenylthiourea, still had much melanin in the pigment epithelium. Nevertheless the autoradiograms

prepared from this series exhibited the same variations as those previously described. Control animals showed high radioiodine level, animals treated continuously with phenylthiourea showed no radioiodine binding, those treated with phenylthiourea and then allowed two days' recovery showed a very low radioiodine level. Yet the degree of pigmentation of the members of all groups was about the same. It is therefore clear that the binding of radioiodine does not depend on the amount of formed melanin present.

3. *Effects of phenylthiourea treatment on radioiodine binding by the horny teeth*

In all larvae given radioiodine a significant localization was found in the horny teeth. The 4-day series (fixed at 6 days after hatching) shows relatively little cornification of the teeth. Nevertheless the autoradiograms indicate that radioiodine was bound in these structures, not only in the controls but also, and apparently to the same extent, in the phenylthiourea-treated animals. The 24- and 39-day series both exhibit extensive cornification of the teeth, and in these there is an indication that phenylthiourea did lessen the binding of radioiodine without, however, completely halting it. Photomicrographs and corresponding autoradiograms of the teeth in control and experimental animals of the 24-day series will serve to illustrate this inhibitory effect (Figs. 7–10). It was found that whereas the autoradiograms of control tadpoles (Fig. 8) and those of treated animals allowed a 48-hour recovery period show equally dense spots, representing the teeth, autoradiograms of larvae subjected to continuous treatment with phenylthiourea show definite but much less intense darkening (Fig. 10). This result was consistent in all specimens of the older series.

4. *Effects of phenylthiourea treatment on the thyroid and thymus*

Although these experiments were not primarily concerned with the thyroid gland, some observations on the thyroid response are of interest. In most of the control larvae of the 4-day series the thyroid proved to be at a stage of early follicle formation. Only one or two follicles were present in each thyroid, the rest of the gland consisting of irregular cords of cells. The formed follicles had a cuboidal epithelium that still retained some of the pigmentation characteristic of the anuran thyroid rudiment. The lumina were very small and contained no stained colloid. Two of the five control specimens had no organized follicles at all, the entire thyroid being composed of clumps and cords of epithelial cells. In the experimental animals of this series, there were some with glands lacking organized follicles; these showed no histological differences from the two controls just mentioned. On the other hand, the phenylthiourea-treated larvae in which follicles had appeared showed a sharp contrast to controls in that the follicles were larger, had a flattened epithelium, and contained a relatively large amount of homogeneous basophilic colloid. The autoradiograms of the control thyroids and those allowed 48 hours' recovery from phenylthiourea showed evidence of binding of radioiodine, whereas the animals treated continuously with phenylthiourea did not. It is noteworthy that the thyroids of all 5 of the controls and of all 5 of the recovery series produced clear autoradiograms but in some of them no organized follicles were yet present. The effect of phenylthiourea treatment on thyroid

physiology is thus detectable histologically as soon as follicles are formed and physiologically even before follicles are formed.

The thyroids of controls fixed at 26 days after hatching showed a quite uniform appearance. The follicular epithelium was cuboidal and the colloid acidophilic, usually with some chromophobe droplets. Experimental animals differed consistently in having many more chromophobe droplets and slightly higher epithelium. The different lengths of treatment with phenylthiourea resulted in no histologically observable differences. There was also no significant difference in the appearance of the thyroids of animals treated continuously and those allowed two days' recovery before fixation. Autoradiograms prepared from the sections of this series showed a high level of radioiodine in the control thyroids, a very low level in the thyroids of larvae treated continuously with phenylthiourea, and a high level, apparently as high as that of controls, in those of larvae removed from phenylthiourea at the time of their exposure to radioiodine solution.

The controls of the series fixed at 41 days after hatching were all in metamorphic stages and, as would be expected, their thyroids gave indications of high activity. The epithelium tended to be columnar and chromophobe droplets were abundant. The experimental animals, on the other hand, had enlarged follicles with a markedly flattened epithelium and much homogeneous colloid. The autoradiograms of this series are similar to those for the 24-day series.

The concentration of radioiodine in the thymus gland observed by Dent and Hunt (1952) was confirmed in these experiments and proved to be affected by phenylthiourea treatment in exactly the same way as is radioiodine concentration in the thyroid. Autoradiograms made from control tadpoles of the 24-day series or from tadpoles allowed a recovery period show high concentration of radioactivity in the thymus; those made from tadpoles under continuous treatment with phenylthiourea show no radioactivity in this region.

Discussion

In these experiments the administration of phenylthiourea to early larvae of *Hyla versicolor versicolor* resulted in the production of completely unpigmented tadpoles. Both concentrations tested (0.01 and 0.005%) proved equally effective and neither gave any indications of toxicity. The gradual blanching of the skin produced by treatment with phenylthiourea has sometimes been referred to as a depigmentation effect. It is probable, however, that the drug has no effect on any pigment already present when treatment is begun but acts entirely by preventing the formation of new pigment. The results of our experiments are in accord with this view for, as has been pointed out, the blanching of the skin (and of the eyes) occurred rapidly in larvae treated immediately after hatching, more slowly in those in which treatment was delayed until 4 days after hatching, and very slowly indeed in those in which treatment was begun 15 days after hatching. It must be assumed that in all these animals the phenylthiourea treatment effectively blocked melanogenesis and that the rate of "depigmentation" depended on the rate of loss of the melanin already present. In fact, it would appear that this rate of blanching after treatment with phenylthiourea should furnish an indication of the normal rate of metabolic turnover of melanin at various ages. Our results indicate that turnover is rapid at early ages but much slower in older animals. In fact, it seems

likely that some of the formed melanin persists indefinitely after a certain age is reached.

These experiments also demonstrate that phenylthiourea affects the binding of radioiodine by the tapetum nigrum. Only the untreated animals show a significant concentration of I^{131} by this structure. Tadpoles given phenylthiourea and then removed from the solution exhibit an ability to bind iodine within the first 24 hours after cessation of treatment. In all experiments, however, the autoradiograms, though they do not give quantitative information, indicate clearly that I^{131} is not taken up by the pigmented epithelium of the eye in direct proportion to the amount of melanin present. Tadpoles for which phenylthiourea treatment is begun at 15 days after hatching retain much pigment in the eye yet show no tendency to bind I^{131}. This indicates that the binding of iodine in the pigmented epithelium of the eye (and doubtless in chromatophores as well) takes place only while melanin is actually being formed and is dependent on some enzymatic activity that is inhibited by phenylthiourea. Since this substance is known to inhibit tyrosinase activity *in vitro* and since tyrosine must be present where melanogenesis is going on, it is natural to suspect that tyrosinase is the enzyme involved. These views are supported by the findings of Gennaro and Clements, who extracted radioactive mono- and diiodotyrosine from discs of frog skin that had been incubated in Ringer solution containing I^{131} and from the skins of intact frogs injected with I^{131} (1956a). They also showed that pretreatment of the discs with thiourea decreased the degree of incorporation of I^{131} in the melanized areas (1956b). According to the concept outlined, tyrosinase would be active in pigment-forming tissues both in the oxidation of tyrosine to melanin and in the oxidation of iodide to iodine to permit the production of mono- and diiodotyrosine and possibly iodinated proteins. Inhibition of tyrosinase activity would thus be expected to result simultaneously in cessation of melanogenesis and failure to bind I^{131}. Whether these findings can be directly related to the goitrogenic effects of phenylthiourea is not certain. The mechanism by which iodination of tyrosine occurs in the thyroid is not well understood (Roche and Michel, 1955). There is no evidence of the presence of tyrosinase in the mammalian thyroid (Pitt-Rivers, 1950). Fawcett and Kirkwood (1954) have hypothecated a "tyrosine iodinase" as a catalyst for the process. There are many varieties of tyrosinase (Lerner and Fitzpatrick, 1950), however, and it may be that amphibian tyrosinase has a special property of oxidizing iodine or, on the other hand, that our experiments may offer the key to a better understanding of iodination of tyrosine in the thyroid itself.

The accumulation of iodine in the horny teeth of larval anurans was first reported by Dent and Hunt (1952). Association of iodine with similar hard structures is known to occur in a number of invertebrates. Noteworthy examples are the hypodermis of Drosophila larvae (Wheeler, 1950), the setae and pharyngeal teeth of polychaetes (Swan, 1950), and the exoskeleton of Daphnia (Gorbman, Clements and O'Brien, 1954). Gorbman (1955) has discussed this matter in some detail and notes that in all these cases the localization of radioiodine is in scleroprotein. The present experiments indicate that phenylthiourea treatment, if given over a sufficiently long period, has an inhibitory effect on the binding of radioiodine here although it does not completely prevent it.

The effects of derivatives of thiourea on the functioning of the thyroid gland have been widely studied in mammals and in several amphibians. The histological

changes seen in the thyroids of the animals studied in our experiments are in agreement with those previously reported and need not be discussed. The autoradiographic analysis of the thyroids of the control and experimental animals also gave results that would be expected on the basis of what is known of the effects of this drug. Larvae under continuous treatment with phenylthiourea showed an extremely low ability to bind I^{131}. However, treated larvae recovered this ability very rapidly after treatment was discontinued. This rapid rate of recovery is in contrast with the slower rate observed in the pigmented regions and may be indicative of a difference in the mechanism of iodine binding.

The basis for the accumulation of radioiodine by the thymus gland is not known. It was first reported by Dent and Hunt (1952), and it is clearly demonstrated in our material. It is completely inhibited in animals under continuous treatment with phenylthiourea but cessation of treatment is followed by prompt recovery of the animal's ability to concentrate iodine. It appears, then, that the binding of iodine in the thymus is more closely related to that process as it occurs in the thyroid than as it occurs in the melanophore.

Earlier observations on various vertebrates (see citations in Dent and Hunt, 1954) have all been to the effect that the onset of iodine accumulation by the thyroid does not occur until discrete follicles make their appearance. It is of some interest, then, that in the animals studied here iodine concentration began while the cells of the thyroid rudiment were still arranged in cords at four days after hatching. Moreover, such animals, when given continuous phenylthiourea treatment, showed no ability to concentrate I^{131}. Thus the iodine-concentrating activity of thyroid tissue, and also the ability of phenylthiourea to inhibit this activity, are evidenced well before the appearance of follicles or colloid.

Summary

1. Larvae of *Hyla versicolor* were immersed in solutions of phenylthiourea at 0, 4, and 15 days after hatching. At 4, 24, and 39 days after hatching, I^{131} was administered and contact autoradiograms were prepared from serially sectioned representative specimens.

2. The tadpoles treated with phenylthiourea from the time of hatching became completely unpigmented. The blanching of the second series was slower and never complete. The third series became very little lighter during the course of the investigation. This indicates that the metabolic turnover of melanin goes on at a decreasing rate as the larvae increase in age.

3. From the autoradiograms, evidence was obtained to confirm earlier findings of the binding of iodine in pigmented areas, to show that the binding is apparently not associated with formed melanin, and to support the view that the same enzyme or enzymes that catalyze melanogenesis can catalyze the binding of iodine (presumably with tyrosine).

4. The accumulation of iodine by the horny teeth was inhibited to some degree by phenylthiourea treatment.

5. Accumulation of radioiodine by the thymus gland was confirmed and was found to be completely inhibited by phenylthiourea treatment.

6. The thyroid rudiment acquires the facility for concentrating iodine even before follicle formation begins, and at that time it also responds to the inhibitory action of phenylthiourea.

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ENDOPARASITIC POLYCHAETOUS ANNELIDS OF THE FAMILY ARABELLIDAE WITH DESCRIPTIONS OF NEW SPECIES[1]

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Among the polychaetes, which for the most part are free-living, crawling, burrowing, and tube-dwelling, commensalism is rather common but internal parasitism is rare indeed. Among the relatively few cases that have been reported are some lumbrinerid-like polychaetes belonging to the superfamily Eunicea, which includes the families Eunicidae, Onuphidae, Lumbrineridae, Arabellidae, Lysaretidae, and Dorvilleidae (sometimes considered as subfamilies belonging to the family Eunicidae). Some of the parasitic euniceans invade other members of the same superfamily and may attain an enormous size in proportion to the host. All of the known lumbrinerid-like parasites belong to, or show affinities to, the family Arabellidae as defined by Hartman (1944).

In connection with a study of polychaete material from various sources, including that in the United States National Museum, some arabellids were found living as endoparasites in other polychaetes of the related family Onuphidae. Two new species are described herein, the types of which are deposited in the United States National Museum. Some small specimens of a third species, living parasitically in the onuphid, *Diopatra*, are thought to be the young stages of the arabellid, *Notocirrus spiniferus* (Moore). Since this interesting type of parasitism is not widely known and has not received the attention it no doubt deserves, the relatively few known cases of lumbrinerid-like species living in other polychaetes and echiuroids are reviewed and the chief characteristics of the Eunicea and Arabellidae to which the parasites belong are summarized.

Superfamily EUNICEA

All the members of the Eunicea are equipped with a complex series of strong, dark, chitinous or horny jaws. The pharynx is capable of protrusion and is provided with a pair of ventral plates, called mandibles, and a more dorsal bilaterally arranged series of plates, called maxillae. The prostomium is distinct, with or without eyes or appendages. Typically the first two segments are apodous. The parapodia are essentially uniramous, the upper lobe or notopodium represented at most by a few embedded notoacicula and a rudimentary papilla-like lobe. The members of the Eunicea vary from those of minute size with a moderate number of segments to some very large ones with very numerous segments, some of which are among the largest of the polychaetes. They are essentially free-living, predaceous, and carnivorous. They secrete abundant mucus, which may aid in burrowing or forming temporary or more or less permanent tubes.

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Family ARABELLIDAE Hartman

The arabellids show a superficial resemblance to the members of the Lumbrineridae, differing from them in setal and pharyngeal characters. They have the body elongate, cylindrical, of nearly uniform width, and tapering slightly anteriorly and posteriorly. The prostomium is reduced to a simple, conical or flattened spatulate lobe, without appendages, with or without eyespots on the posterior margin. The first two segments are distinct, apodous, and without appendages. The parapodia are essentially uniramous; the notopodia sometimes represented by a minute papillar lobe (sometimes referred to as a reduced dorsal cirrus) with embedded notoacicula; the neuropodia are unequally bilobed, with shorter rounded and longer digitiform postsetal lobes. The neurosetae are simple (not compound), limbate, and taper to fine tips; in addition they sometimes have projecting thick

TABLE I

Parasite[1]	Host and Distribution	References
Notocirrus sp. (young)	Eunicidae: *Marphysa sanguinea* (Montagu). Mediterranean	Koch, 1847. Ehlers, 1868, p. 364. See below
Notocirrus ?spiniferus (Moore) (young)	Onuphidae: *Diopatra cuprea* (Bosc). Woods Hole region, Massachusetts	Allen, 1952. See below
Haematocleptes terebellidis Wirén	Terebellidae: *Terebellides stroemii* Sars. Off Sweden, 130 meters	Wirén, 1886
Labrorostratus parasiticus Saint-Joseph	Syllidae: *Odontosyllis ctenostoma* Claparède, *Syllis prolifera* Krohn, *Eusyllis monilicornis* Malmgren, *Pionosyllis lamelligera* Saint-Joseph, *Grubea clavata* (Claparède)	Saint-Joseph, 1888. Caullery and Mesnil, 1916. Fauvel, 1923, p. 440
Oligognathus bonelliae Spengel	Echiuroidea: *Bonellia viridis* Rolando. Mediterranean	Spengel, 1882. Fauvel, 1923, p. 442
Oligognathus parasiticus Cerruti	Spionidae: *Spio mecsnikowianus* Claparède. Mediterranean	Cerruti, 1909. Fauvel, 1923, p. 442
Drilonereis parasiticus (Caullery)	Terebellidae: Genus and species? Near Timor, Dutch East Indies, 73 meters	Caullery, 1914. See below
Drilonereis forcipes (Hartman)	Eunicidae: *Eunice* sp., possibly *E. antennata* Savigny. San Benito Island, Lower California, 66–81 fathoms	Hartman, 1944. See below
Drilonereis benedicti n. sp.	Onuphidae: *Onuphis magna* (Webster). Tampa Bay, Florida, 12 fms.	See below
Drilonereis caulleryi n. sp.	Onuphidae: *Onuphis* (*Nothria*) *conchylega* Sars. Off Massachusetts to off Virginia, 101–317 fms.	See below

acicular setae or acicula (without hooded hooks as in the Lumbrineridae). The parapodia have no dorsal or ventral cirri or branchiae. The eversible proboscis is equipped with strong, chitinous, black jaw pieces: usually with a pair of ventral, flat plates, the mandibles; with 4 or 5 pairs (may be fewer in parasitic forms) of more dorsal maxillae arranged in parallel rows, with a pair of long filiform carriers to which a shorter median unpaired piece is attached on the ventral side. The arabellids are essentially a burrowing, predaceous, carnivorous group. They burrow readily but rather slowly in sand or mud. They secrete a good deal of mucus, which probably serves to lubricate the burrow.

Some of the arabellids are parasitic in other polychaetes (eunicids, onuphids, syllids, terebellids) and in echiuroids (*Bonellia*), living inside the body cavity or vascular body wall or even in the vascular system of the host, at least during their

early developmental stages. The eight previously reported parasitic arabellids, along with the two species described herein, are summarized in Table I and in the illustrated Key to the genera and species.

Illustrated Key to the Genera and Species of Parasitic Arabellidae

[Figures are copied from the original descriptions. a, anterior end; b, parapodium; c, setae; d, mandibles; e, maxillae and maxillary carriers; f, maxillae]

A¹. Numerous specimens in single host.

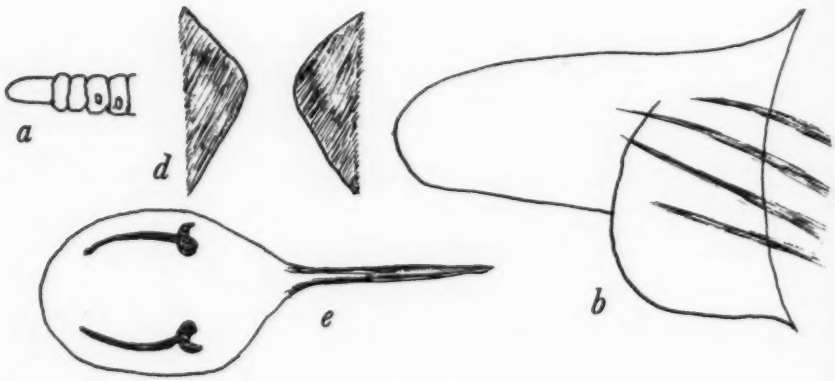
B¹. Parasitic in body cavity of *Marphysa sanguinea* . . . *Notocirrus* sp. (young). (See below under *N. spiniferus.*)

B². Parasitic in body cavity and vascular body wall of *Diopatra cuprea* *Notocirrus ?spiniferus* (young). (See Figs. 4, g and 5.)

A². One parasite per host.

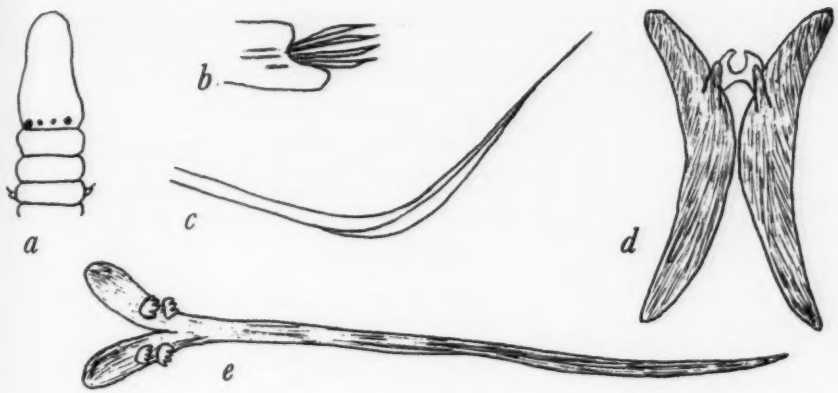
C¹. One to three pairs of rudimentary maxillae, with single elongate rodlike maxillary carrier. Mandibles present.

D¹. Maxillae a single pair of curved rods, each curved tooth at base. Setae and acicula not projecting from lobe. Mandibles paired, triangular. Prostomium without eyes. (Up to 25 mm. long, about 200 segments. Colorless.) Parasitic in peri-intestinal blood sinus of *Terebellides stroemii*. . . . Genus *Haematocleptes* Wirén *H. terebellidis* Wirén



D². Two or three pairs of maxillae. Setae projecting from parapodial lobe.

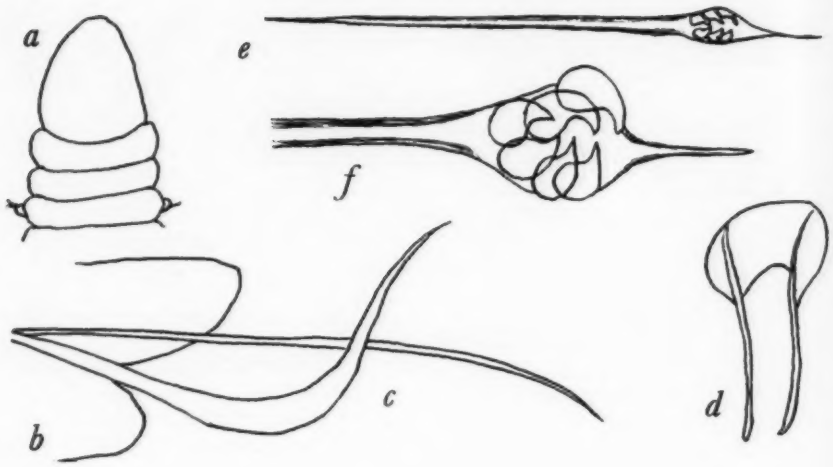
E¹. Maxillae two pairs of very small denticled pieces. Mandibles wing-shaped, each with a spine. Setae all of one kind, limbate, smooth, tapering to long flexible tips. Prostomium with four eyes in transverse row. (Up to 70 segments.) Parasitic in body cavity of syllids, *Odontosyllis ctenostoma, Syllis prolifera, Eusyllis monilicornis, Pionosyllis lamelligera, Grubea clavata.* Also found free among calcareous algae, *Lithothamnion.*
Genus *Labrorostratus* Saint-Joseph *L. parasiticus* Saint-Joseph



F². Maxillae three pairs recurved unidentate hooks. Mandibles U-shaped, with two wing-like pieces or rods united by transverse band. . . . Genus *Oligognathus* Spengel

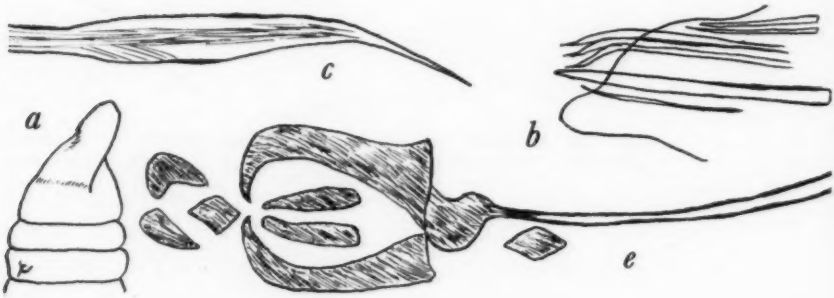
F¹. Setae of one kind, simple, arched, limbate, striated. Prostomium with four eyes. (Up to 100 mm. long, more than 200 segments. Bright orange yellow.) Parasitic in body cavity of echiuroid, *Bonellia viridis*.
O. bonelliae Spengel

F². Setae of two kinds: capillary, flexible and stouter, wide, tapering to fine tips. Prostomium without eyes. (Up to 8 mm. long. 50 segments. Colorless, transparent.) Parasitic in body cavity of spionid, *Spio mecznikowianus*.
O. parasiticus Cerruti



C². Four pairs of well developed maxillae, with pair of elongate rodlike maxillary carriers and shorter unpaired piece (basal or maxillae I large, strong, hooked, forceps-like; maxillae II rectangular plates, sometimes denticled; maxillae III and IV each a single strong thorn-like tooth). Mandibles absent. Parapodia with heavy acicula or acicular setae, the tips of which usually protrude. Prostomium without eyes. Genus *Drilonereis* Claparède (includes *Labidognathus* Caullery; see below).

G¹. Parasitic in peri-intestinal blood sinus of terebellid (unidentified). With bilimbate setae and single stout acicular seta extending out of parapodial lobe. Maxillae II edentate (?, incompletely observed). (More than 100 segments). . . . *D. parasiticus* (Caullery)



G². Parasitic in body cavity of branchial fragment of eunicid, *Eunice* sp., possibly *E. antennata*. With bilimbate setae and single stout yellow aciculum, the latter not extending out of parapodial lobe. Maxillae II flat plates, practically without teeth. (More than 140 segments, more than 30 mm. long.). . . . *D. forcipes* (Hartman)

G[3]. Parasitic in body cavity of onuphids. Maxillae II rectangular plates, each with four distinct teeth.

H[1]. Parasitic in branchial fragment of *Onuphis magna*. Without setae or acicular setae visible externally (even in a specimen of more than 1200 crowded segments and more than 240 mm. long). . . . *D. benedicti* n. sp. See Fig. 1, a-g.

H[2]. Parasitic in anterior fragment of *Onuphis* (*Nothria*) *conchylega*. With limbate setae and single stout acicular seta extending from lobe (except in smaller specimens; up to 400 segments, 110 mm. long). . . . *D. caulleryi* n. sp. See Fig. 2, a-o.

The parasitic arabellids may be separated into two main groups. The first includes those species of which numerous specimens are found in a single host. Within a single host, they may be found in varying stages of development, from small specimens, with few segments, no eyes, and no jaws, to larger ones, with numerous segments, eyes, developing jaws, parapodia, and setae. The two reported cases are thought to be the young stages of species of *Notocirrus*. When first observed by Koch (1847), they were reported as a lumbrinerid-like stage of the young of the presumably viviparous eunicid host, *Marphysa sanguinea;* this species lives in a loose mucous tube, irregularly encrusted with rocks, shells, and such. Similar forms have since been found in the onuphid, *Diopatra cuprea,* which lives in a parchment-like tube, one end of which is buried in the sand or mud, the other end sticking out of the substratum and covered with shells, plant debris, etc. The parasites evidently penetrate the host at an early stage, just how early and the mechanism for penetration being unknown. They grow and develop within the host to an advanced stage, when they evidently leave the host and perhaps continue to grow and mature, taking on a free-living existence.

The other type of parasitism is the condition where a single parasite is found in a host and where the parasite may attain enormous dimensions in comparison to the host, becoming nearly as large or larger than the host or host fragment. They evidently penetrate the host at an early stage also and grow to an advanced stage, perhaps even completing their growth within the host. The parasite may be completely enclosed in the host or part of it may protrude. Perhaps it matures after leaving the host, as sex products have not been observed in the parasites found in the host.

The parasitic arabellids appear to be rare. Most of the records of the different species have been based on single specimens. In a long study from 1875 to 1888 at Dinard, Saint-Joseph (1888) observed only 14 examples of *Labrorostratus parasiticus* in the body cavities of several species of syllids. After numerous years of microscopical examination of numerous syllids, Caullery and Mesnil (1916) observed only a single parasite of the same species. Considering that the syllids are small and somewhat transparent and that the parasites may be as much as three-fourths of the length of the host, the presence of the parasites would probably be

noted. Where the host is larger and opaque, the parasites would be observed only accidentally in fragmented or dissected specimens.

Four of the parasitic species belong to the genus *Drilonereis,* which also includes free-living species. The maxillae and maxillary carriers are well developed. In *D. caulleryi* n. sp. the parapodial armature develops gradually; first the heavy aciculum appears, then the setae, only the tips of which project at first, then the setae project further and the tips of the heavy acicula finally protrude. In *D. benedicti* n. sp. the acicula and setae are rudimentary and do not project from the parapodial lobes, even in a specimen of more than 1200 crowded segments. The parasitic genera, *Oligognathus* Spengel, *Labrorostratus* Saint-Joseph, and *Haematocleptes* Wirén, have the maxillae more rudimentary than in *Drilonereis,* with a single elongate rodlike maxillary carrier, darker toward the outside. *Haematocleptes terebellidis* shows the most rudimentary condition, having only a single pair of maxillae and the setae and acicula not projecting from the parapodial lobe.

Genus *Drilonereis* Claparède, 1870

Type (by original designation): *D. filum* (Claparède, 1868).
Labidognathus Caullery 1914; type (by monotypy): *L. parasiticus* Caullery, 1914.

Diagnosis. Prostomium conical to spatulate, flattened ventrally, usually with central depression dorsally, without eyes or appendages. First two segments apodous and achaetous, first sometimes partially fused dorsally to prostomium. Parapodia with dorsal lobe or notopodium usually small, rudimentary, with a few embedded notopodial acicula; neuropodium with two unequal lips, supported by acicula. Setae all simple, of two kinds: (1) bilimbate or winged, tapering to fine tips, smooth or faintly striated (not denticled); (2) 1–2 stout acicular setae with tips protruding from parapodial lobe; (in some parasitic forms, setae may be rudimentary, not extending out of parapodial lobes). Pharynx with mandibles or lower jaws rudimentary or absent; maxillae or upper jaws 4–5 pairs, symmetrical, dark, chitinous, supported by a pair of long slender maxillary carriers and a shorter unpaired piece; basal maxillae I large, heavy, falcate pincers or forceps; maxillae II rectangular plates, usually denticled; maxillae III and IV with one to few teeth; maxillae V rudimentary or absent.

Remarks. Labidognathus is herein referred to *Drilonereis.* The type species of the former, *L. parasiticus* Caullery (1914, p. 490), was found living as a parasite in a terebellid (not yet described) near Timor, Dutch East Indies; the parasite was found in the peri-intestinal blood sinus, coiled in a complicated manner around the intestine of the host. According to Caullery, both the host and parasite were in rather poor condition; the jaw apparatus of the parasite was not studied completely. Hartman (1944, p. 180) noted the affinities of *Labidognathus* with *Drilonereis* and described a new species, *L. forcipes,* found in the body cavity of a fragment of a species of *Eunice* from San Benito Island, Mexico. In addition two new species are described below. Because of the scarcity of material of the parasites, it is difficult to work out the developmental stages. As indicated below for the four specimens of *Drilonereis caulleryi* n. sp., parasitic in *Onuphis conchylega,* there are differences in the development of the jaws and parapodia in different stages of growth. The four parasitic species of *Drilonereis* show essentially the characters

of the genus. They all lack mandibles and have four pairs of maxillae, of which maxillae I are stout falcate hooks and maxillae III and IV each a single stout conical hook. The parasites were found singly, one to a host. The hosts, at least when collected, were either anterior or middle fragments, with the parasites protruding and exposed in part.

Drilonereis benedicti n. sp.

Fig. 1, A–G

The species is known from a single specimen, incomplete posteriorly (U.S.N.M. No. 28637), found in a fragment of 18 segments from the branchial region of

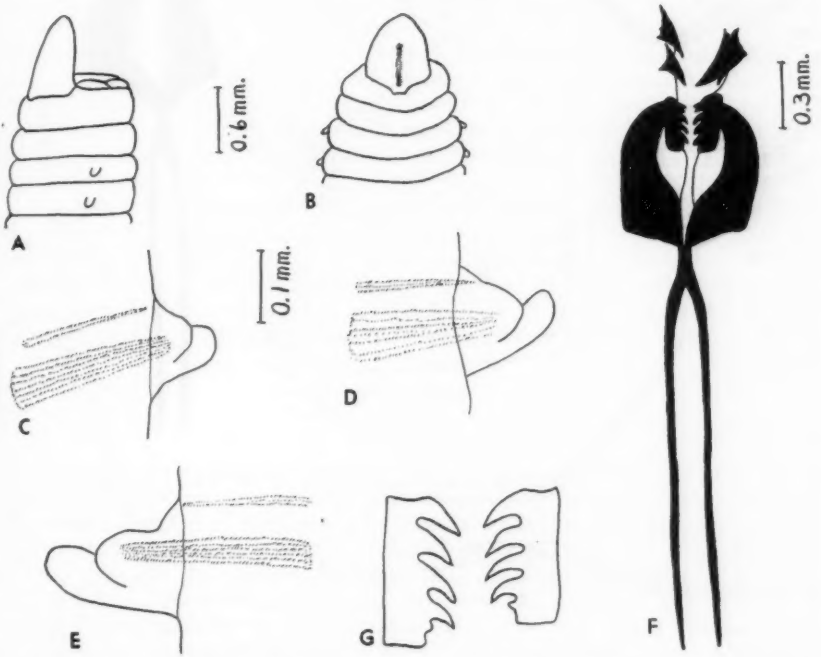


FIGURE 1. *Drilonereis benedicti* n. sp.: A, Lateral view anterior end; B, dorsal view anterior end; C, left parapodium from setiger 10, anterior view; D, same from about setiger 300; E, right parapodium from about setiger 600, anterior view; F, four pairs maxillae and maxillary carriers, dorsal view (more ventral unpaired piece not shown); G, maxillae II, enlarged.

Onuphis magna (Andrews), North Channel into Tampa Bay, Florida, 12 fms., *Fish Hawk* Sta. 7108, 1901, Dr. J. E. Benedict, collector. The parapodia of the host fragment were compared with those of an incomplete specimen of *O. magna* found at the same station. The middle part of the parasitic *Drilonereis* extended through the body cavity of the host fragment, the greater part of the anterior and posterior

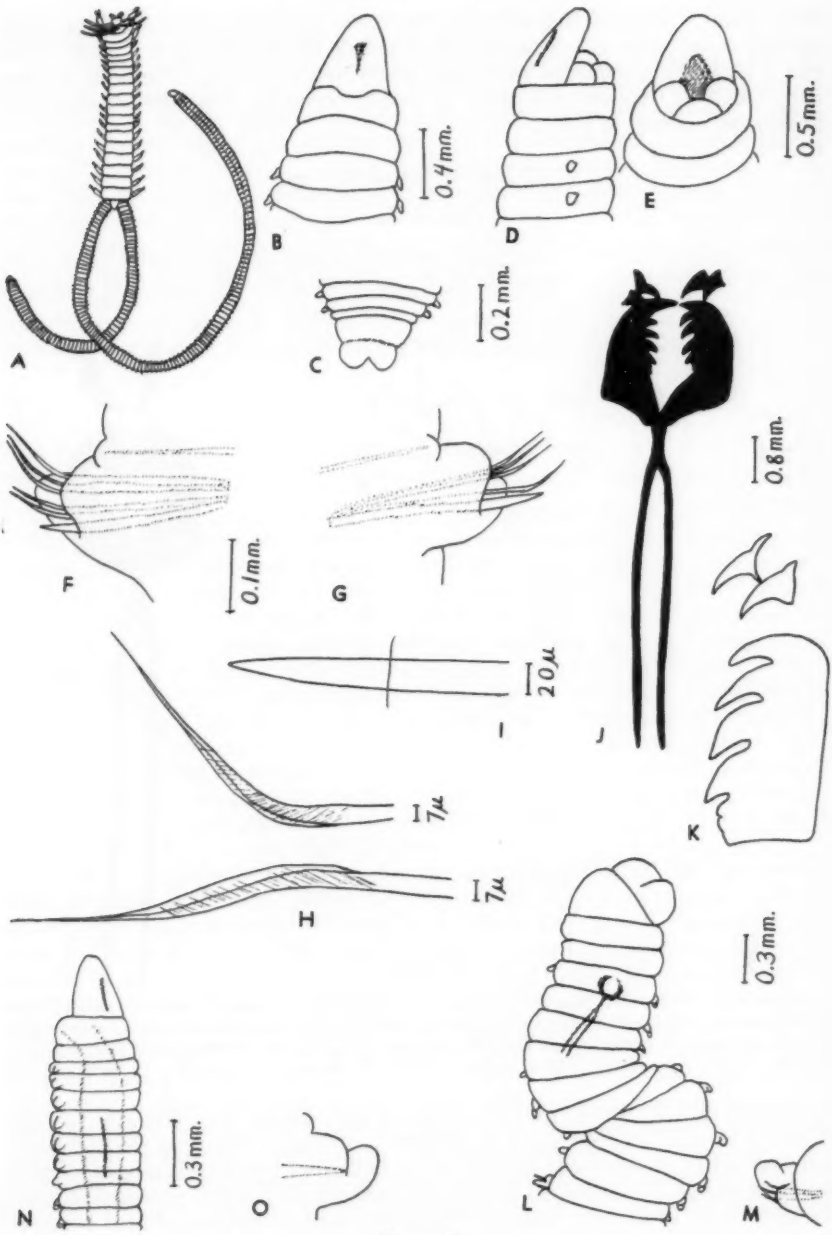

A
B
0.4mm.
C
0.2mm.
D
E
0.5mm.
F
0.1mm.
G
0.8mm.
I
20μ
J
K
17μ
H
17μ
0.3mm.
N
0.3mm.
O
L
M

FIGURE 2.

ends of the parasite being exposed. It is named for the collector, who evidently put it aside to be worked up later.

Description. Length of incomplete specimen 240 mm., greatest width 1.5 mm., segments more than 1200. Body cylindrical, with segments slightly longer anteriorly becoming very short and crowded posteriorly, colorless (in alcohol), shiny iridescent anteriorly, dull posteriorly. Prostomium (Fig. 1, A, B) conical, rounded anteriorly, flattened ventrally, with a longitudinally depressed area mid-dorsally. First two segments achaetous, subequal to the following, first with mid-dorsal nuchal notch. Parapodia (Fig. 1, C–E) similar along length of body, small, unequally bilobed, with shorter rounded and longer thick digitiform lobes. No setae exposed external to lobes; internally few notopodial acicula extending into base of rudimentary low notopodial lobe and larger group of neuropodial acicula and setae with tips extending into the short neuropodial lobe; one of the acicular group is much stouter, probably corresponding to the stout acicular seta characteristic of *Drilonereis*. Proboscis without mandibles and with four pairs maxillae. Maxillae (Fig. 1, F, G) well developed, dark, with a pair of long, dark filiform maxillary carriers and short oval unpaired piece, dark anteriorly, light amber-colored posteriorly; basal maxillae I stout, falcate, hooked; maxillae II rectangular plates, each with four distinct teeth and slight indication of a fifth; maxillae III and IV each a single, large, thorn-like tooth.

Remarks. D. benedicti differs from the other parasitic species of *Drilonereis* in the complete absence of exposed setae, even in a specimen of more than 1200 segments. The host normally lives in a parchment-like tube. Perhaps the host fragment moves along in the tube carrying the parasite with it. Possibly the parasite at this stage feeds for itself, as the anterior and posterior ends were exposed. There is also the possibility that the host fragmented at the time it was collected.

Drilonereis caulleryi n. sp.

Fig. 2, A–O

The species is represented by four specimens, each of which was found living parasitically in anterior fragments of *Onuphis* (*Nothria*) *conchylega* Sars, collected by the *Fish Hawk* and *Albatross* off Martha's Vineyard, Massachusetts, and off Cape Henry, Virginia, from 1880 to 1883. Two of the four specimens are small, showing different developmental stages. The specimen designated as the type (U.S.N.M. No. 12867) is complete and was found coiled inside a host of about 40 anterior segments. The largest paratype (U.S.N.M. No. 8987) consists of an anterior end of 25 mm. and a posterior end of 10 mm. protruding from the posterior

FIGURE 2. *Drilonereis caulleryi* n. sp.: A, Habit sketch of parasite and host (2); B, dorsal view anterior end (1); C, dorsal view posterior end (1); D, lateral view anterior end, proboscis partially extended (2); E, ventral view same; F, left parapodium from setiger 20, anterior view (1); G, right parapodium from middle of body, anterior view (1); H, limbate setae from same; I, acicular seta from same; J, four pairs maxillae and maxillary carriers, dorsal view (more ventral unpaired piece not shown) (2); K, right maxillae II–IV, enlarged; L, dorsal view anterior end of slightly coiled smaller paratype, proboscis partially extended (3); M, parapodium from setiger 10 of same; N, slightly lateral view anterior end of smallest paratype (4); O, parapodium from setiger 12 of same. (1) type specimen; (2) largest paratype specimen; (3) smaller paratype specimen; (4) smallest paratype specimen.

end of the host fragment of 18 segments, the middle part of the parasite being inside the host (Fig. 2, A). A smaller paratype (U.S.N.M. No. 28636) occupied setigers 8–18 of an anterior fragment of a host of 18 segments; the posterior end of the parasite protruded from the posterior end of the host fragment. A very small paratype (U.S.N.M. No. 28635) was found in a host fragment of 17 segments; the anterior end of the parasite was sticking out dorsally between setigers 15 and 16, the posterior end was protruding ventrally between the same segments. The species is named for Dr. Maurice Caullery, who described the first parasitic drilonereid (as *Labidognathus*).

Description. Length up to 110 mm., greatest width up to 1 mm., segments up to 400 or more. Body cylindrical, shiny iridescent. Prostomium (Fig. 2, B, D, E) conical, rounded anteriorly, flattened ventrally, with a mid-dorsal depressed area. First two segments apodous and achaetous, subequal to the following, first with mid-dorsal nuchal notch. Anal end (Fig. 2, C) short, cylindrical, tapering to pair of short bulbous lobes (no distinct anal cirri). Parapodia (Fig. 2, F, G) similar along length of body, short, unequally bilobed, with shorter rounded and longer, thick digitiform lobes. Two larger specimens with two kinds of setae projecting from parapodial lobe: 4–5 bilimbate, straight and curved setae, tapering to slender tips, faintly striated (not denticled; Fig. 2, H); also single yellowish stout, pointed acicular seta (Fig. 2, I); with additional internal neuropodial acicula as well as few notopodial acicula, the tips of which extend into short bulbous rudimentary notopodium; posterior end of body with tips of limbate setae only projecting from parapodial lobes. Smaller specimen (12+ mm. long, 0.6 mm. wide) with tips of limbate setae only extending out of lobe, stout acicular seta being visible inside lobe (Fig. 2, 1, M). Smallest specimen (5 + mm. long, 0.3 mm. wide) with no setae projecting, single stout acicular seta being visible inside parapodial lobe (Fig. 2, N, O). Proboscis, when partially extended, appearing as three bulbous lobes (Fig. 3, D, E); no mandibles; maxillae four pairs, well developed, dark, with pair of long filiform carriers and shorter wide oval unpaired piece, dark anteriorly, lighter more posteriorly (Fig. 2, J, K); maxillae I stout, falcate, forceps-like; maxillae II rectangular plates, each with four distinct teeth; maxillae III and IV each a single large hooked thorn-like tooth. In smaller specimen, maxillary forceps and slender carriers visible through transparent body wall (Fig. 2, L). In smallest specimen (Fig. 2, N) only maxillary carriers visible.

Remarks. D. caulleryi differs from the other parasitic species of *Drilonereis* as indicated in the key above. As shown in the three developmental stages found in the four specimens, the maxillary carriers develop first, then the stout maxillary forceps; in the parapodia, the stout acicular seta appears first, then the limbate setae, the tips of which may protrude, the stout acicular seta not protruding (this would correspond to the condition described for *D. forcipes* by Hartman); finally the limbate setae protrude further and the tips of the stout acicular setae protrude. The host, *Onuphis conchylega* Sars, occupies a flat parchment-like tube encrusted with flattened pieces of rocks and shells. All four host specimens are anterior fragments. The parasite may be completely enclosed in the host fragment or a portion of the parasite may stick out, revealing its presence. The parasite perhaps gets into the host by encouraging it to fragment; none of the fragments showed any signs of regeneration, although *O. conchylega* fragments and regenerates readily.

Material examined. Type: off Martha's Vineyard, Massachusetts, 39° 53′ N., 69° 47′ W., 317 fms., soft green mud, *Fish Hawk* Sta. 1096, 1882. Paratypes: 40° 02′ N., 70° 23′ W., 115 fms., mud, fine sand, *Fish Hawk* Sta. 871, 1880; 40° 02′ N., 70° 37′ W., 101 fms., grey mud, fine sand, *Fish Hawk* Sta. 1108, 1882; off Cape Henry, Virginia, 37° 19′ N., 74° 26′ W., 102 fms., green mud, shell, *Albatross* Sta. 2004, 1883.

Distribution. Atlantic, off Massachusetts to off Virginia. 101 to 317 fathoms.

Genus *Notocirrus* Schmarda, 1861, emend. Ehlers, 1868

Type (designated by Ehlers, 1868, p. 406): *N. chilensis* Schmarda, 1861.

Diagnosis. Prostomium conical, without appendages, usually with 4 eyespots. First two segments apodous and achaetous. Parapodia with dorsal lobe or notopodium represented by small rudimentary lobe and few notopodial acicula; neuropodium unequally bilobed, supported by stout acicula, the tips of which project (except in young developing stages). Setae all simple, bilimbate or winged, tapering to fine tips, striated and finely to coarsely denticled along limbate border. Pharynx with pair of wing-shaped, dark chitinous mandibles or lower jaws; maxillae or upper jaws 4–5 pairs, dark, chitinous, denticled, supported by pair of long filiform maxillary carriers and shorter unpaired piece; maxillae I and II asymmetrical, maxillae I dentate throughout entire length or only slightly falcate or with short hook distally.

Remarks. *Notocirrus* has affinities with *Arabella,* having similar prostomia and pharyngeal jaws; both have limbate setae with denticled border. *Notocirrus* also has affinities with *Drilonereis,* both having stout acicula or acicular setae which project from the parapodial lobes. A species of *Notocirrus* is herein reported to be parasitic in an onuphid (*Diopatra*) during its early development.

Notocirrus spiniferus (Moore, 1906)

Figs. 3–5

Arabella spinifera Moore, 1906, p. 501, pl. 19, figs. 1–7.

The species was described from a single specimen which was dredged on muddy bottom in the middle of Buzzards Bay, Massachusetts. No additional records have been reported. In working over a good deal of material collected in New England and vicinity, four additional free-living specimens were found. The species appears to be rare but, due to its superficial resemblance to the more common *Arabella iricolor,* it may be confused with that species in collections. The four specimens were obtained from the following sources: Buzzards Bay, Massachusetts, 1956, H. Sanders, collector; off Cape Henry, Virginia, Chesapeake Bay, 9 fms., shelly and sand, *Fish Hawk* Sta. 8838, 1920; Isle of Wight Bay above Ocean City, Maryland, 1953, S. McDowell, collector; Beaufort, North Carolina, 1951, E. Cole, collector.

In addition two of the specimens found living parasitically in the body cavity of *Diopatra cuprea* (Bosc), collected by M. Jean Allen at Hadley Harbor, Nonamesset Island, Woods Hole region, Massachusetts, were examined. They ap-

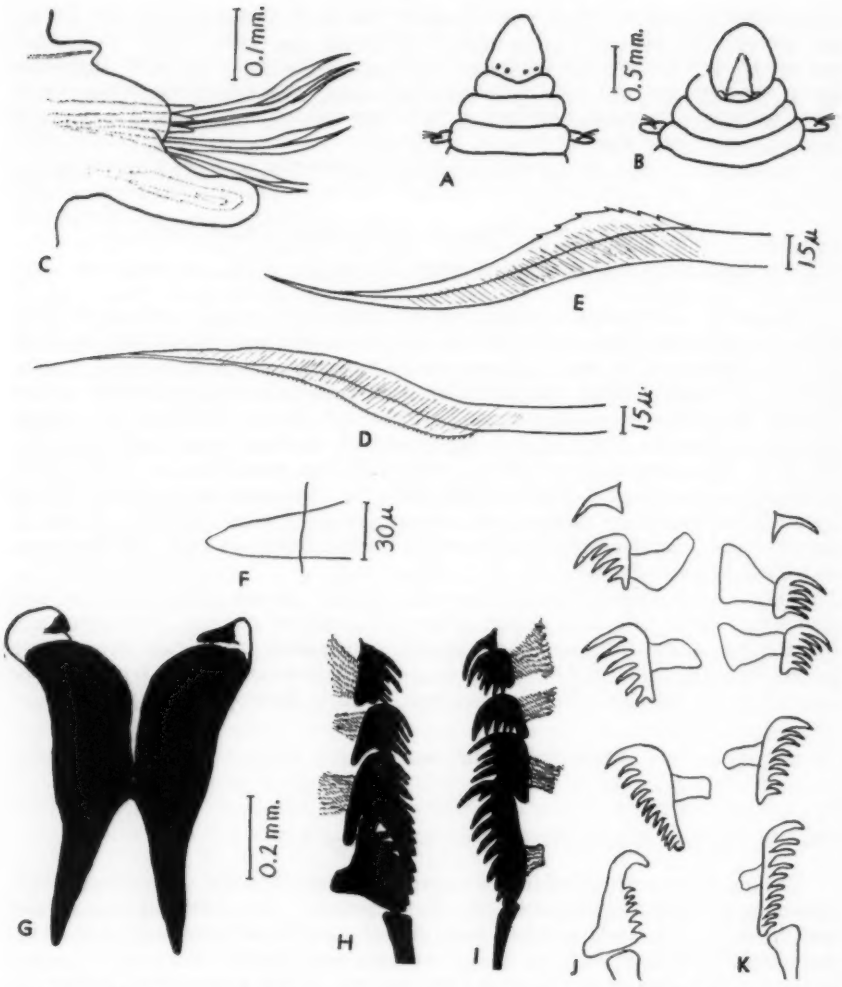


FIGURE 3. *Notocirrus spiniferus.* Drawn from specimen from Buzzards Bay: A, Dorsal view anterior end; B, same, ventral view; C, parapodium from setiger 10, posterior view; D, limbate seta from same; E, limbate seta from setiger 100; F, tip of one of acicula from same; G, mandibles, ventral view; H, left maxillae, I–IV, dorsal view; I, right maxillae, I–IV, dorsal view; J, left maxillae spread apart; K, right maxillae spread apart.

pear to be the young of a *Notocirrus*, possibly that of *N. spiniferus*. In a note recording the find of more than 50 parasitic specimens in a single specimen of *Diopatra*, Allen (1952) indicated that they might be the young of *Arabella iricolor* but the parapodia differ from that species as indicated below.

Description of specimens found free-living. Length 40–110+ mm., width 1–4 mm., segments 140–220+. Body cylindrical, tapering slightly anteriorly and posteriorly, stiff, wiry. Prostomium (Figs. 3, A, B; 4, A, B) subconical, rounded anteriorly, slightly depressed dorsoventrally but not greatly flattened as in *Drilonereis;* a pair of faint longitudinal grooves ventrally and four eyes in transverse row at posterodorsal border, rather than two eyes as reported by Moore. First two segments achaetous and apodous, first with mid-dorsal nuchal notch. Parapodia (Figs. 3, C; 4, D) prominent, similar along length of body, with small but distinct notopodium supported internally by few notoacicula; neuropodium bilobed, with

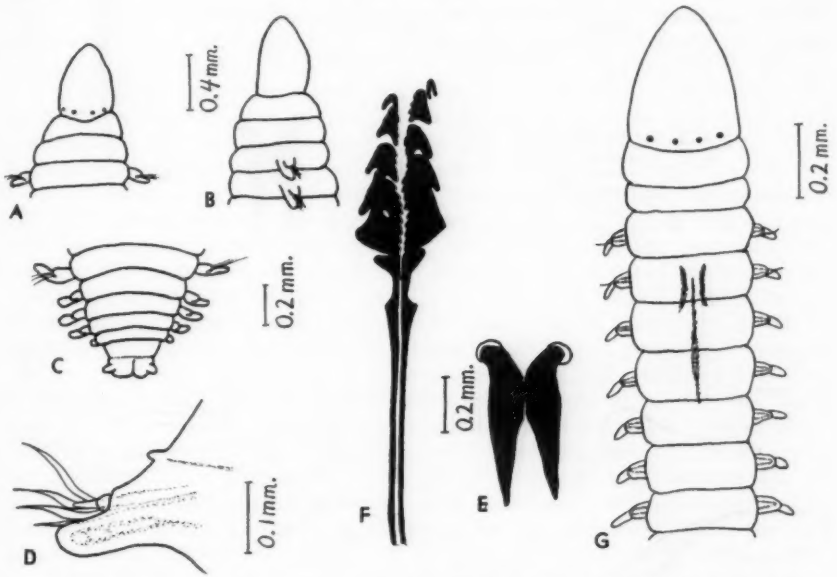


FIGURE 4. *Notocirrus spiniferus.* Drawn from specimen from Isle of Wight Bay (A–F): A, Dorsal view anterior end; B, lateral view same; C, dorsal view posterior end; D, parapodium from middle of body; E, mandibles, ventral view; F, five pairs maxillae and maxillary carriers, dorsal view (more ventral unpaired piece not shown). G, Dorsal view anterior end of small specimen of *Notocirrus ?spiniferus,* living parasitically in *Diopatra cuprea,* Woods Hole region.

short rounded setal lobe and longer, prominent digitiform postsetal lobe (sometimes referred to as ventral cirrus or cirriform branchial organ), within which is a vascular loop. Setal lobe with 1–3, usually 2, stout, deep yellow acicula, the tips of which project out of lobe (thus differing from *Arabella,* which has no stout projecting acicula); acicular tips bluntly pointed, tapered abruptly to short fine tips, or sometimes obviously broken (Fig. 3, F). Setal lobe with 4–8 bilimbate, doubly curved setae with fine tips, with wings wide, striated and finely denticled along border, sometimes with a few coarser denticles near base of wing (Fig. 3, D, E). Anal end (Fig. 4, C) tapered to pair of short bulbous lobes, each with very short rudimentary anal cirrus.

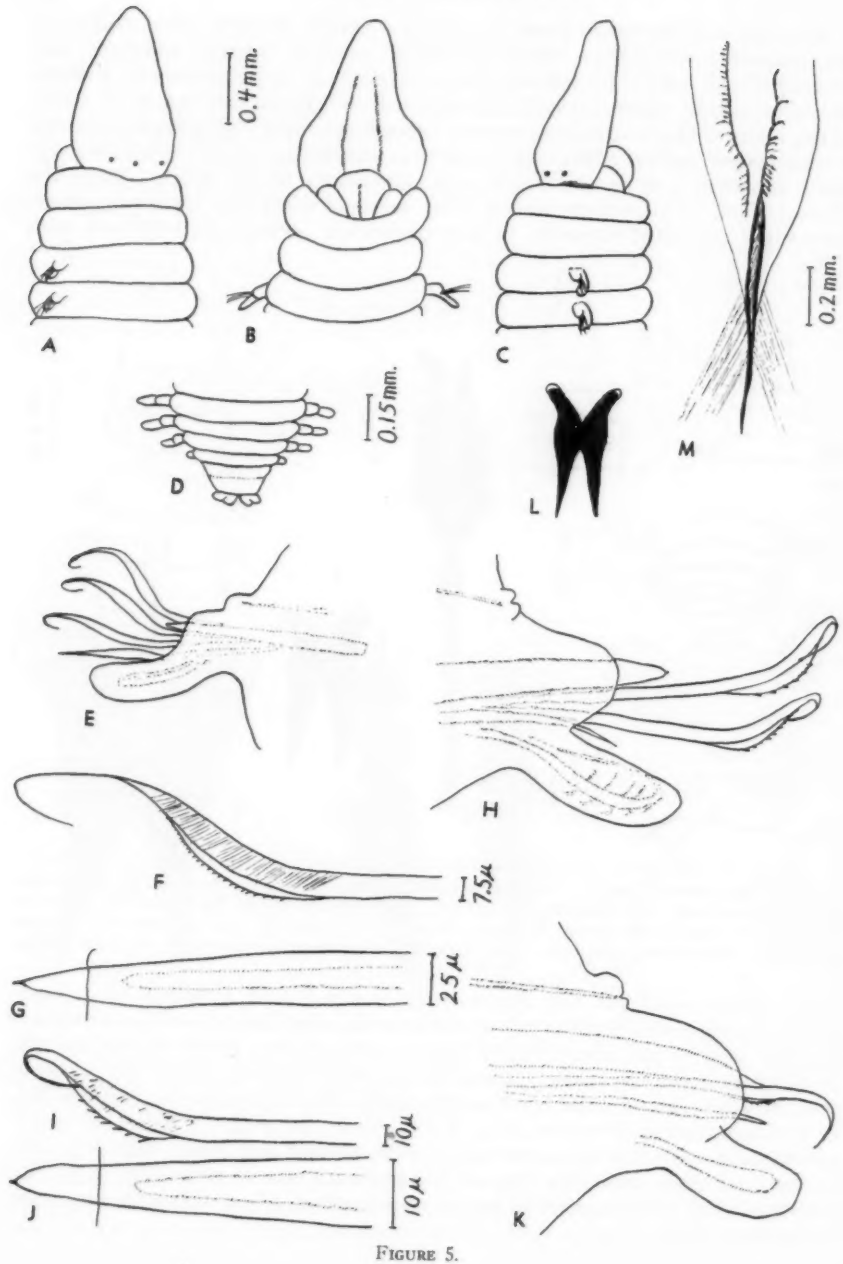


FIGURE 5.

Proboscis, when partially extended, appears as bulbous bilobed tongue with pair of lateral lobes. Ventral mandibles (Figs. 3, G; 4, E) well developed, brown or black, wing-shaped, sometimes with exposed white tips. Five pairs of dorsal jaws or maxillae (Figs. 3, H–K; 4, F), with pair of long slender carriers, thickened distally and subdistally, and shorter unpaired piece; fifth pair, consisting of single tooth, easily confused with the fourth, to which it is closely allied. First two or basal pairs of maxillae asymmetrical. Right maxilla I longer than left, with up to 10 denticles along length of inner border and without distal hook; left maxilla I with up to six basal teeth and distinct distal hook. (Moore, in figure of the type, showed distal hooks on both first maxillae and fewer teeth.) Left maxilla II much larger than right, completely overlapping left maxilla I and extending down to maxillary carriers, with up to 12 or 13 teeth; right maxilla II partially overlapping right maxilla I, with up to 8 or 10 teeth. Maxillae III to V symmetrical, III each with 6 teeth, IV each with 5 teeth, and V each a single tooth, without basal extension as in other maxillae. (The last may appear to blend in with maxillae IV and this may have led to Moore's count of only four pairs of maxillae.) Color (in alcohol) yellowish to brownish, iridescent.

Description of two young specimens of Notocirrus ?spiniferus, living parasitically in Diopatra cuprea. The larger parasitic specimen is 25 mm. long, 1.5 mm. wide, and consists of almost 200 segments. The smaller specimen is 12 mm. long, 0.5 mm. wide, and contains about 150 segments. Prostomium of smaller one (Fig. 4, G) conical, bullet-shaped, that of larger one (Fig. 5, A–C) more elongated, bulbous basally; both show four eyes in transverse row. Parapodia of smaller specimen showing characteristic bilobed form, without setae projecting except for first two setigerous segments in which a single seta projects; each of setal lobes provided with stout aciculum (appears dark basally). In larger specimen, parapodia of anterior region (Fig. 5, E) with four limbate setae and single stout aciculum; those of middle region (Fig. 5, H) with two limbate setae and tip only of a third one projecting; those of posterior region (Fig. 5, K) with single limbate seta and tip of a second one projecting; notopodia small but distinct, with tip of notaciculum extending into lobe. Limbate setae with long fine tip, curled backward, distinctly denticled along limbate border (Fig. 5, F, I); stout projecting acicula with fine short tips (Fig. 5, G, J). Thus parapodia essentially as in larger free-living specimens of *Notocirrus spiniferus* except for fewer acicula and setae. Anal end of small specimen tapering gradually to cylindrical posterior end, without distinct anal cirri; that of larger specimen (Fig. 5, D) essentially as in *N. spiniferus*. Pharynx, when partially extended, appears as three-lobed structure, the middle lobe rounded, tongue-like, consisting of pair of lobes (Fig. 5, B). Jaw parts of smaller specimen (Fig. 4, G) not dissected but darker paired mandibles and maxillary carriers visible through the somewhat transparent body wall, maxillary carriers appearing as single elongated rod. Jaw parts of larger specimen, when dissected out, showing well

FIGURE 5. Young *Notocirrus ?spiniferus*, parasite taken from body cavity of *Diopatra cuprea*, Woods Hole region: A, Laterodorsal view anterior end; B, ventral view same; C, lateral view same; D, dorsal view posterior end; E, parapodium from setiger 10; F, limbate seta from same; G, aciculum with projecting tip from same; H, parapodium from middle of body; I, limbate seta from same; J, aciculum with projecting tip from same; K, parapodium from posterior part of body; L, mandibles, ventral view; M, developing maxillary carriers and maxillae, dorsal view.

developed mandibles (Fig. 5, L), and incompletely developed maxillae (Fig. 5, M) with elongated rodlike maxillary carriers (appearing as single rod but darker toward the outside), and developing denticled maxillae, indicated by slightly darker amber-colored areas on walls of pharynx.

Remarks. The smallest parasites reported by Allen were composed of seven segments; some specimens of 30 segments showed no eyes or setae; the largest specimen reported was 50 mm. long and was composed of about 200 segments. These parasites had not emerged naturally from the host. Thus they live a parasitic life for a considerable period. The smaller specimens were in the body wall of the host near the parietal blood vessels, the larger ones were free in the body cavity. A few large parasites were observed emerging from the body cavity of *Diopatra* by Dr. Frank Brown (Allen, 1952), but the lengths and the developmental stages of these specimens were not indicated. It may be that the parasites remain in *Diopatra* until the jaw parts are completely developed; they were not completely developed in the largest specimen I was able to examine. It is unknown how the parasites get into the host. Perhaps the eggs of *Notocirrus* are laid and fertilized within the tube of *Diopatra*. *Notocirrus*, being a burrowing form, could enter the parchment-like tube of *Diopatra* on the buried end which, as far as has been observed, is open. In some way, the fertilized eggs or young at a very early stage get into the body cavity of *Diopatra*. The posterior end of *Diopatra* is soft and flaccid and fragments easily; perhaps the young are able to bore into the broken fragmented end of the host. *Diopatra* regenerates readily also. One host, found by Allen, contained about 30 small parasites, composed of from 7 to approximately 30 segments; another host contained over 50 parasites of varying sizes, some up to 50 mm. in length.

In this connection, it may be of interest to mention the observation made by Koch (1847) of filamentous lumbrinerid-like forms crawling out of a broken truncated posterior end of a strongly contracting specimen of *Marphysa sanguinea*, which was dredged at considerable depth in the Mediterranean. On further examination of the *Marphysa*, he found numerous young specimens in the body cavity, in various stages of development; the smallest were small roundish microscopic forms with only slight indication of a few segments and without eyes; a more advanced stage of 25–30 segments showed a distinct prostomium with 2 eyes, parapodia with stout aciculum only, and jaw apparatus in early stages of development; a still more advanced stage of 50–100 segments showed a distinct prostomium with 4 eyes in a transverse line, parapodia with 2 stout acicula and a few setae confined to the parapodia; a still later stage of 100–120 segments showed the parapodia with a short rounded setal lobe and a longer postsetal lobe, with stout dark yellow acicula, the tips of which appear from Koch's figure to project, as in *Notocirrus* or *Drilonereis*, and the jaw apparatus well formed (probably not completely formed). Koch thought that the specimens he observed were the young stages of a viviparous *Marphysa sanguinea* and that during its development, the young pass through a lumbrinerid-like stage. Ehlers (1868, p. 364), commenting on the observation of Koch, indicated that Koch had more likely observed parasitic forms of a lumbrinerid-like species living in *M. sanguinea* and that the latter was not viviparous; he stated that it was unreasonable to think that a young specimen of *M. sanguinea* of more than 100 segments would not show some of the characteristics of the adult, that of antennae, branchiae, characteristic setae, etc. In consideration

of the long jaw pieces (maxillary carriers) and the four eyes, Ehlers concluded that it might be a parasitic species of *Arabella*. The figures and description given by Koch suggest to me that the young developing stages in *Marphysa* that he observed, were the parasitic young stages of a species of *Notocirrus*, as indicated especially by the long maxillary carriers, the stout parapodial acicula, and the four eyes in a transverse row.

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A DIURNAL ACTIVITY RHYTHM IN PLETHODON CINEREUS AND ITS MODIFICATION BY AN INFLUENCE HAVING A LUNAR FREQUENCY

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It has become increasingly clear that many of the physiological processes in organisms do not occur at constant rates, even when the organism is in a constant laboratory environment. These fluctuations in rates are often of regular recurrence and may be designated as rhythms.[2] Various manifestations of these changes taking place within the organisms may be observed. Among them are rhythms of O_2-consumption and CO_2-production, locomotor activity, chromatophore pigment dispersal and body temperature changes.

Judging from the number of contributed works in the field of biological rhythms, locomotor activity has been more often utilized as an index to rhythmic behavior than any other kind of biological process. The simplicity of automatic recording devices needed, the long span of time over which animals may be used for such studies, and the minimal interference with the animals' normal functioning, are some of the reasons why activity studies have been so popular.

Among the earliest investigators of activity rhythms was Szymanski (1918), who demonstrated them in a variety of animals. Subsequently, studies of the activity rhythms of a host of animals have been made. Among them, to name only a few representative ones, are those of Ralph (1957) on the earthworm, Harker (1956) on *Periplaneta americana*, Brown (1954) on the oyster, Marx and Kayser (1949) on lizards, and Aschoff (1952) on mice.

The diurnal rhythm is the one most commonly encountered in terrestrial organisms (reviewed by Welsh, 1938, and Kleitman, 1949). Among marine organisms both diurnal and tidal rhythms have been found associated together (Brown, Fingerman, Sandeen and Webb, 1953), but sometimes a tidal rhythm may be the only one apparent (Rao, 1954). Lunar periodicities in marine animals are well known (Korringa, 1947).

That lunar influences may also be significant in the metabolic rhythms of terrestrial organisms has been indicated by recent works (Brown, Freeland and Ralph, 1955; Ralph, 1957). The present study was undertaken in order to examine the activity behavior of a terrestrial animal, the salamander, *Plethodon cinereus*, and to analyze it for lunar influences.

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[2] A rhythm, as used here, is defined as a definitely persisting, regularly recurring, quantitative change that continues after external stimuli are withdrawn. Rhythms can be roughly divided into (a) those of 24 hours (diurnal) or less, and (b) those of more than 24 hours (*e.g.*, lunar). They generally have a causal relation to external factors, but are to be distinguished from periodicities which are of extrinsic origin and which vary directly with environmental factors.

Experimental Procedure

Twenty-four adult *Plethodon cinereus* were collected on May 8, 1955, in a beech-maple forest near New Buffalo, Michigan. The animals were brought to the laboratory and placed in two 9-inch crystallizing dishes, the bottoms of which were covered with a moist sand layer and bits of wood debris. Small earthworms and sowbugs were provided as food and these were replenished frequently during the experiment. The dishes were placed in a slowly-moving stream of cold tap water.

All work was carried out in a room designed as a photographic darkroom. There was one opening to the room, a door fitted with a light-baffle. Thermograph records of the air temperature a few inches above the running water in which the animals were kept throughout the experiment were taken between May 10th and 26th. These showed no diurnal temperature variations, but rather only slow changes requiring several days.

Continuous records of the locomotor activity of seven animals were obtained by the use of the same apparatus, and data were translated from the experimental records to tabular form in the same manner, as described by Ralph (1957) for the study of the earthworm. The recording device consisted essentially of seven rectangular platforms that rocked freely upon knife-blade bearings. Upon each platform was mounted a chamber consisting of a 3¾-inch Petri dish enclosing the bottom half of a 2¾-inch Petri dish, thereby forming a circular track one-half inch in width. Movement of the animal around this track resulted in different degrees of tipping of the platform. Platform movements were transmitted via a thread to a recording pen system that reproduced them on a sheet of paper moving at a rate of two centimeters per hour. The recording apparatus occupied a position adjacent to the dish of reserve animals and the activity chambers were suspended about two inches above the water's surface in order to keep the animals in the same cool air layer.

From May 9th to 13th all the animals were exposed to alternating light, from 6 A.M. to 6 P.M., and darkness, from 6 P.M. to 6 A.M. The light source during this period was two 7½-watt opalescent, incandescent lamps suspended about four feet above the animals. The form of the diurnal activity cycle under simulated day and night conditions was determined during this period.

During the afternoon of May 13th a light-proof box was mounted over the water table in which the reserve animals were kept so that they could be maintained in constant low illumination. The box was equipped with a light source at the top so that diffuse light of less than one foot-candle reached the salamanders. Also, the transparent glass covers of the activity chambers were replaced by black-painted covers in order to exclude all light. Thus, when the animals were in the reserve dishes they were under continuous and constant low illumination, and when placed in the recording apparatus they were in darkness. Twenty-nine days of continuous records for seven animals at a time were obtained under these conditions.

A regular sequence of replacement was established at the outset of the experiment, so that only one or two animals were replaced daily. As the animals were removed from the chambers they were placed in one of the crystallizing dishes, while the replacement animal was randomly selected from the other dish. When the supply of animals was exhausted from one dish, replacement was started from the

dish that had been receiving the animals. Thus, each of the 24 animals participated in the study at least twice for approximately four days each time.

In order to minimize any effects that the placing of a fresh animal in the apparatus might have on the data, the time of replacement was varied over the day from 8 A.M. to midnight. As a further precaution, the first three hours of the record produced by a fresh animal were not included in the data, since the animals tended to be hyperactive for several minutes after being placed in a chamber.

Results

Diurnal rhythm

The mean activity for the animals in alternating light and darkness is shown by Figure 1, A.[3] It will be seen that the animals were most active during the

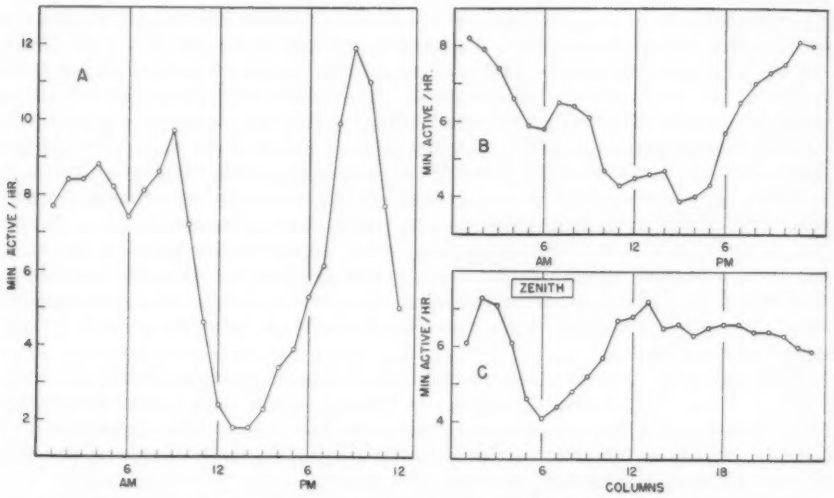

FIGURE 1. (A) The average activity of seven salamanders for about five consecutive days while exposed to light from 6 A.M. to 6 P.M. and darkness from 6 P.M. to 6 A.M. (B) The average activity of seven salamanders for 29 consecutive days. Their activity was recorded while they were in darkness. (C) The influence of the lunar-day frequency on the activity of the salamander, as demonstrated by analysis for lunar effects. (See text for explanation.) The times of zenith for the 29-day period were synchronized in column 6 for this analysis.

dark hours and least active during the light hours. A more complete analysis of the exact form of this curve will be presented in the discussion.

Table I shows the mean activity when the animals were in dark chambers. These data represent hourly determinations for 29 consecutive days. The mean values for seven animals for each hour were placed in the table under the hour on which the determination ended.

[3] All graphs in this paper are plotted from sliding averages of three adjacent values. For example, the averages of the 2, 3, and 4 P. M. columns were averaged to give the 3 P.M. value. This technique is useful for smoothing curves and shows trends more clearly.

TABLE 1

Locomotor activity in the salamander (min./hr.)

	A.M.												P.M.											
	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
May 14	3.6	1.9	7.9	1.9	0	0.3	5.7	5.7	6.0	1.1	6.7	5.0	4.0	0	0.7	1.4	1.4	5.6	1.0	8.6	12.9	8.6	6.4	0.3
15	0.4	5.0	9.6	0.3	0.3	5.7	0.3	0.4	4.3	0.7	6.7	5.0	0.3	0.3	0.7	3.6	6.0	12.1	15.7	22.4	5.7	11.7	3.6	7.4
16	10.7	11.0	7.1	5.0	11.1	7.1	0	0.6	1.7	0.3	1.4	1.4	6.1	6.2	3.3	7.0	7.0	8.5	5.8	0.7	8.6	6.7	11.0	22.1
17	10.7	8.6	4.9	7.9	2.1	3.1	0	4.3	5.0	4.6	0.8	4.6	1.4	0	0.3	0	2.1	0.4	3.6	5.7	1.4	0	7.1	7.9
18	4.6	5.0	8.6	7.4	0	5.0	8.9	1.0	4.3	4.3	2.4	0.3	8.6	10.0	3.3	1.0	0	0	16.7	9.7	5.3	2.4	8.9	8.6
19	8.4	11.0	8.6	0	2.1	2.9	0	7.1	1.4	0	3.7	0	0	0	0.7	0	2.0	7.0	1.5	4.3	5.0	9.3	8.0	4.0
20	4.3	1.9	4.4	12.9	5.3	7.9	16.4	8.1	1.7	2.9	0.7	8.6	1.4	0.3	0	0	0.7	7.1	5.3	8.6	0.4	0	1.9	13.6
21	9.3	7.9	5.0	8.6	12.9	13.6	9.3	27.9	16.4	10.0	0	0.4	8.6	7.9	0	0	0	0	0	0	4.6	10.0	8.9	10.7
22	8.9	15.0	17.0	8.6	11.4	9.3	2.9	3.6	3.6	0.3	4.5	0.8	0.5	5.7	1.4	0.7	0	6.4	6.4	9.3	11.1	8.6	7.1	16.0
23	12.1	7.9	8.6	2.1	2.4	6.0	0.7	11.0	10.0	7.5	4.2	9.3	2.6	2.9	7.9	5.8	0.3	0.7	5.7	7.6	12.5	1.2	0.7	0
24	4.1	0	4.3	9.3	5.0	7.4	9.4	0.6	6.4	0.4	0.3	2.0	0	8.3	10.7	2.1	0.6	2.4	17.4	13.2	4.3	12.1	11.4	0
25	0	1.7	3.6	8.6	5.7	5.0	10.7	4.4	0	6.4	0.3	4.3	0	0	0	0	8.6	8.6	15.3	10.3	18.3	25.0	11.0	19.3
26	17.4	19.3	6.4	0.4	13.6	14.6	4.3	0	0.7	0.3	12.5	14.2	0	2.9	7.9	0.7	16.6	20.0	20.3	17.9	23.1	20.0	15.0	23.6
27	4.3	0	3.1	12.9	14.3	8.9	8.9	0.3	6.4	0	0	3.1	12.5	10.0	6.7	8.9	12.1	1.4	13.6	15.3	2.1	12.5	4.2	10.8
28	16.4	13.2	7.1	2.4	6.7	0	0.3	0	0.7	1.4	1.0	5.0	9.3	9.3	12.9	10.0	0	0	19.2	16.7	14.6	12.1	6.9	5.3
29	13.9	31.4	18.1	12.9	8.6	7.7	5.0	14.3	11.3	8.0	3.3	7.9	10.0	0.7	4.3	8.6	1.4	7.1	5.7	10.0	7.9	17.1	5.0	10.7
30	13.6	2.1	6.1	8.6	11.4	4.6	0	8.6	7.1	7.9	4.3	18.3	17.5	2.8	0	0	0	0	1.4	0.7	0	0.8	0	11.6
31	17.1	13.9	2.1	10.7	7.1	3.8	8.6	16.4	5.7	0.7	5.0	13.6	6.0	3.8	12.1	2.1	15.0	20.0	7.5	7.9	0	0	0	0
June 1	4.2	12.9	12.1	4.3	3.8	2.1	7.1	13.9	11.7	13.9	5.0	1.4	1.4	2.1	5.3	0	5.7	3.6	0	0	2.9	2.8	11.7	10.0
2	3.3	0	13.6	8.6	1.4	0	14.3	11.4	10.3	14.3	2.9	3.6	7.9	3.3	17.5	10.3	11.4	8.6	5.3	0.7	3.6	3.6	0	5.0
3	5.7	8.1	10.7	6.4	9.6	2.1	4.3	15.7	6.4	0	0	0	13.6	10.0	2.9	0	0	2.9	8.9	1.7	0	0	4.2	5.0
4	0	19.3	11.4	2.9	2.1	2.9	13.6	17.1	18.0	18.3	8.8	2.0	0	0	7.1	7.1	13.6	14.3	9.3	8.6	3.6	0	5.7	0
5	4.3	5.3	0	0	7.1	22.9	12.1	14.3	2.1	5.3	8.6	8.6	5.3	8.3	5.3	0	0	0.3	0	2.1	25.6	16.2	4.2	8.6
6	4.6	2.1	6.4	1.4	4.6	6.4	4.3	17.9	10.7	9.1	2.9	3.3	0.3	0	5.7	0	0.6	0	0	0	3.6	8.6	9.6	9.5
7	3.3	10.0	14.3	9.3	3.6	3.6	0	0	0	5.3	9.3	8.6	18.6	10.0	10.8	10.0	15.0	21.4	8.6	0.8	0	9.2	15.0	6.0
8	8.6	3.6	0	0	5.7	18.6	12.1	16.4	5.7	6.4	0	1.4	9.3	3.6	5.8	2.8	0	0	0	2.1	7.9	18.6	15.0	1.4
9	2.1	0.7	12.9	8.6	5.7	0.7	0.7	4.3	3.8	0	0	0.3	1.0	9.5	5.8	0	1.2	0	6.7	0	2.1	0.7	18.9	18.6
10	4.6	7.1	15.0	10.0	3.6	0.3	3.6	3.0	3.1	6.2	3.7	0.2	3.0	1.0	0.3	0.9	4.3	2.9	2.9	6.4	13.6	10.0	17.5	12.8
11	15.0	11.4	5.7	0.7	2.1	2.1	1.9	0.3	0	3.6	6.4	0.7	0.3	0.2	1.0	0.3	3.1	0	5.7	7.1	5.7	1.4	0	11.4

The data were first analyzed to find the mean activity for each hour of the day. All 24 columns were averaged and the results are plotted in Figure 1, B. The form of this variation is similar, in a general way, to that obtained when the animals were exposed to alternating light and darkness. There are conspicuous differences between them, however, and these will be discussed later.

Lunar analysis

The data of Table I were inspected for a lunar influence before they were subjected to a detailed analysis. First the data for each day were plotted, each day under the preceding one, and then a line representing lunar zenith was drawn across the plots, intersecting the abscissae at the time of zenith. Since the moon reaches zenith for any given locality approximately 50 minutes later each day, the line representing zenith intersected the time scales about 50 minutes later with each successive day.

Upon close examination of these plots, it was seen that the activity pattern for each day had certain unique variations, but generally bore a similarity to the mean pattern for the 29 days. However, it was noted that when zenith occurred at times of usually moderate or high activity the level of activity was generally low for a few hours before and after the time of zenith. That is to say, when zenith occurred during the "night hours," which were usually the times of greatest activity, depression of activity resulted.

On the starting day of this 29-day study, May 14th, the moon was in third, or last, quarter and thus the time of zenith was approximately 6 A.M. On May 21st new moon occurred with the time of zenith at noon. First quarter, with zenith at approximately 6 P.M., was on the 28th of May and full moon, with zenith at midnight, occurred on June 5th.

It may be postulated that the mean activity for any one day would tend to be high if zenith occurred during the times of normally low activity and would tend to be lower if zenith occurred during the times of high activity. Therefore, if one plots the mean activity for each day for one lunar month, the resulting curve should be essentially the inverse of the mean hourly activity curve, provided the lunar effect is operative in the postulated manner. A comparison of Figure 2, which shows the daily means for a lunar month, with Figures 1, A or 1, B, the hourly mean curves, bears out this hypothesis. This would then suggest strongly that a lunar zenith effect is operative; it is a depressive effect, and the time at which zenith occurs does indeed appear to determine to some extent the mean activity for any one day.

A second method of demonstrating the presence of an apparent lunar modification of the diurnal rhythm was applied in the following manner. If the hourly averages of the days between third quarter and new moon are found, a pattern of activity variation essentially like the average for the entire 29 days should result, since little depressive influence should be in evidence during this time. Figure 3, A, shows the means for each hour of the day for the seven days of that period, and it will be seen that this pattern is very similar to that for the entire 29-day period. Likewise, the hourly means for the succeeding seven days, May 21st to 27th, that is, from new moon to first quarter, should also be similar to the means for the entire 29 days and similar to the pattern for the preceding seven days. Upon examination of Figure 3, B, it will be seen that this is true.

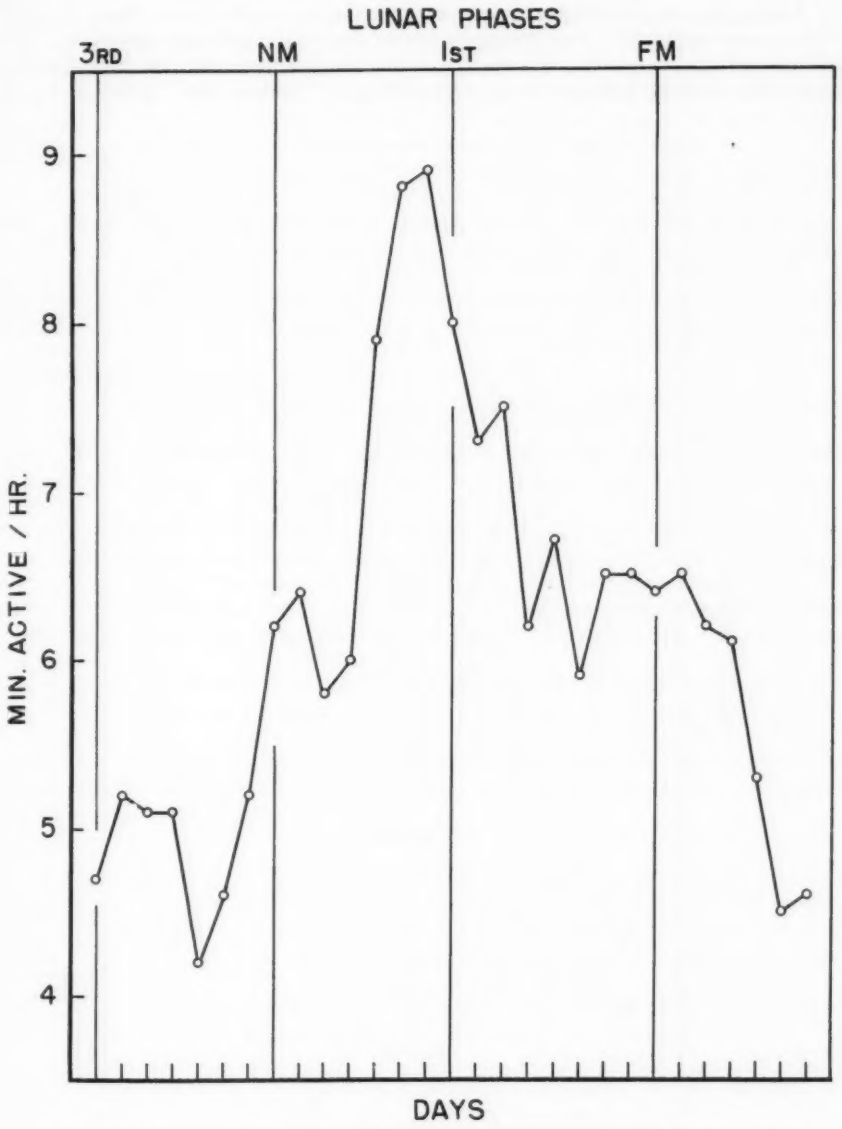

FIGURE 2. The average activity per day for seven salamanders over a 29-day period. The approximate times of the lunar quarters are indicated.

During the next eight days, however, the time of zenith moved from about 6 P.M. to near midnight. Any zenith-associated depressive influence should be very evident during this period. Figure 3, C shows that for these days the lowest levels of activity occurred between 6 P.M. and midnight. Evident, also, in this figure is

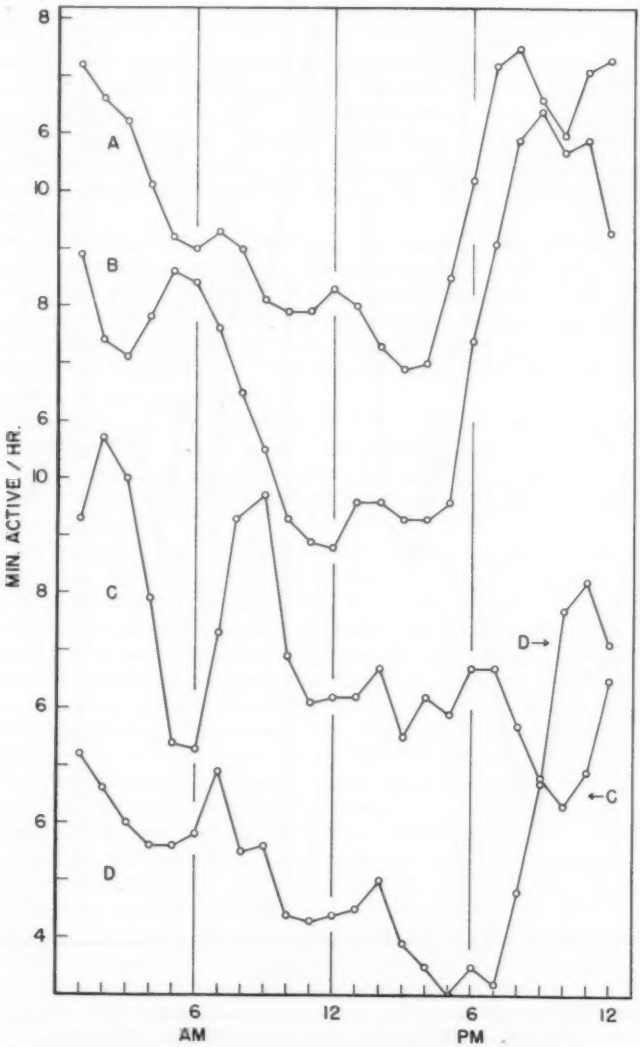

FIGURE 3. The average hourly activity of seven salamanders. A. May 14–20 (3rd quarter-New Moon). B. May 21–27 (New Moon-1st quarter). C. May 28-June 4 (1st quarter-Full Moon). D. June 5–11 (Full Moon-3rd quarter).

a low at 6 A.M. This was largely caused by a low average value of 2.9 at 6 A.M. Consequently, the sliding averages in the region around 6 A.M. are affected. Its significance is unknown.

Finally, the hourly averages for the last quarter of the lunar month are shown in Figure 3, D. Once again, depression appears evident, this time between 1 and 6 A.M., the range over which zenith occurred during the final seven days of this study.

The third method for showing the presence of a lunar rhythm is that which has been employed with much success by Brown (Brown, Bennett and Webb, 1954). For the purposes of this analytical technique, one may visualize the times of lunar zenith, and other corresponding lunar positions, as diagonals running downward and from left to right across the daily rows of data in Table I. As pointed out earlier, the time at which lunar zenith occurs for any one location is later with each successive day by about 50 minutes. Thus, the lunar day is of about 24.8 hours duration. A given lunar position completes one diagonal crossing of all the 24-hour vertical columns in about 29 days.[4]

Any lunar-associated influence may be made apparent if the corresponding lunar positions are aligned in vertical columns. Such a manipulation will serve also to "neutralize" the diurnal rhythm. To accomplish this, the day-by-day data of Table I were shifted to the left an average of about 50 minutes with respect to the preceding day. That is, day 1 was left in its normal hourly relationship, as was day 2, also. Then days 3 to 7 were each moved one hour to the left with respect to the clock hours of the preceding days. The data of day 8 were kept synchronized with those of day 7, but days 9 to 13 were each shifted one hour further to the left, and so on throughout the 29 days of data. Twenty-four vertical columns were retained by transposing, in sequence, the data which extended to the left beyond the first value of day 1 to the right side of the table.

Figure 1, C, shows the results of this analysis. Since zenith occurred at approximately 6 A.M. on the initial day of this experiment and all zeniths of the succeeding days were aligned with it, the position of zenith is indicated in the sixth column in Figure 1, C. This analysis provides further evidence that a depressive effect, which modulates the diurnal rhythm, is associated with the time of lunar zenith.

Discussion

Upon comparison, it will be observed that the amplitude of the average cycle represented in Figure 1, A is about twice that shown by Figure 1, B. This difference may be explained upon the basis of two possible reasons, both of which may apply. When the animals are in alternating light and darkness (Fig. 1, A), the persistent rhythm may be amplified due to a direct influence of light intensity on locomotion. In continuous darkness the rhythm may fail to attain fullest expression, being "damped," but maintaining essentially the same frequency.

A second contributing cause for the difference in amplitude, and one which, in addition, may explain the occurrence of the minor minimum around midnight in Figure 1, A, could be that, in the period represented, only the first half of the A.M. hours, approximately, were subjected to the postulated depressive lunar influence, whereas all hours of the period represented by Figure 1, B were exposed to this

[4] The synodical lunar month, the period from one new moon to the next, has a mean length of 29 days, 12 hours, 44 minutes, and 2.8 seconds.

influence. Thus, Figure 1, B presents the average diurnal rhythm with the lunar influence "neutralized," but nevertheless with lowered amplitude caused by the lunar depression. Figure 1, A is possibly distorted, in part, by the lunar influence, but most of its values were little affected by lunar depression.

The rhythm of locomotor activity, as determined in this experiment under constant laboratory conditions, is undoubtedly similar to the variations in activity of this salamander in nature. Park, Lockett and Myers (1931) note that in the forest this salamander apparently passes the day beneath logs and stones and becomes active by 8:45 P.M.

The possession of a rhythm that assists in regulating the activity of the salamander, as described here, may be of supreme importance for the survival of the animal. As we have seen, it appears to have an activity rhythm that is determined by two components: (1) the diurnal activity pattern, which tends to keep activity minimal during the daylight hours and maximal during the night hours, and (2) the lunar modulation, which alters the diurnal pattern so as to minimize activity on moonlit nights. Thus, the inference is that the animal forays out of its hiding niche on nights when there is little or no moonlight and, hence, when there is less exposure to predators.

Since the period of study extended through only one lunar cycle, it cannot be unequivocably stated that the lunar-frequency modulation constitutes a lunar rhythm, but due to its close correlation with the lunar cycle and the similarity of this modulation with lunar rhythms that have previously been described (*cf.* Brown, Freeland and Ralph, 1955), it appears very likely that it is a lunar rhythm.

Though there appears to be a causal relationship between the lunar cycle and the lunar modification, there need be no direct inductive influence of the moon affecting the organisms. Diurnal rhythms show a causal relationship to the day-night cycle, but, as several studies have shown, the phase relationships of rhythms to the day-night cycle need not be fixed. Thus, the apparent "influence" of the moon on the activity rhythm may be only a persisting behavioral pattern that continues after the inductive influence of the moon is removed. Just as the 24-hour solar cycle may be impressed upon the activity pattern, likewise the 24.8-hour lunar cycle may also be impressed upon the pattern. The two frequencies together would appear largely to determine the overt expression of activity.

The author wishes to express his appreciation to Dr. F. A. Brown, Jr., for his helpful suggestions concerning the manuscript.

Summary

1. The salamander, *Plethodon cinereus*, shows a diurnal rhythm of locomotor activity, both in alternating 12-hour periods of light and darkness, and in continuous darkness.

2. The diurnal rhythm is strongly modified by a depressive influence that is apparently associated with the time of lunar zenith.

3. The activity of the animal at any given time is a function of the diurnal and lunar influences operative at that time.

4. The significance of the rhythm to the animal in nature is discussed.

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STUDIES ON THE EFFECTS OF IRRADIATION OF CELLULAR PARTICULATES.[1] II. THE EFFECT OF GAMMA RADIATION ON OXYGEN UPTAKE AND PHOSPHORYLATION

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It is now well established that dilute solutions of many enzymes are readily inactivated by ionizing radiation and that the presence of solute molecules, other than enzyme, decreases the effect of the radiation (Barron, 1954). Since the classical experiments of Dale (1940, 1942), it has been possible to explain the effects of low dosages of radiation on the basis of interactions between the protein molecule and the ionization products of water. These findings have led to the discovery of important facts about radiation damage and about the nature of enzymes. However, an important question is raised by this work; that is, are we able to draw valid conclusions about the biological effects of radiation from such studies? It is evident that cellular enzymes are not in a pure state, nor are they as dilute as is necessary to achieve effects in some cases. This makes it necessary to investigate the effects of radiation on enzymes under conditions which approximate those of the cell.

There are two obvious ways to do this. The most usual method is to radiate a whole organism (or cell) and then determine the enzymatic activity after radiation. The results of such work indicate that the damage to enzymes by lethal doses of radiation may be negligible (LeMay, 1951). However, there are a number of obvious difficulties in such work; and since the organisms do die eventually and do show loss of respiration in some cases (Barron, 1954), one is left with an unsatisfied feeling. For this reason, it seemed advisable to study the effects of ionizing radiation on cellular particulates (Yost, Robson and Spiegelman, 1956). The particulates of intermediate size (mitochondria) offer several interesting possibilities for such investigations: they can be isolated from the cell in good condition; they contain a large number of vital enzymes; they have a definite structure to which some of the enzymes are attached; the enzyme studied would always be in an environment similar to, if not exactly the same as, that in which it finds itself within the cell; and most important, the particulate is sufficiently large that one might assume that a major part of the damage done to enzymes within the particulate would result from the passage of the ionizing "particle" through the mitochondrion itself. This would mean that the effects of the radiation on the enzyme studied would be the same whether the mitochondrion was extracted or within the cell.

The experiments reported in this paper had a two-fold purpose: the establishment of a dose-inactivation curve for an enzyme known to be closely associated with the structure of the particulate, and the determination of the effect of ionizing radiation on the phosphorylation mechanism. Cytochrome oxidase was chosen as the

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test enzyme since it is known to be closely bound to the particulate and therefore might be an indicator of effects of the radiation on the particulate as a whole, and since it is of vital importance to the electron transport system. Furthermore, the implication of the cytochrome system in the production of mutations by ionizing radiation (Haas *et al.*, 1954) suggests this enzyme as an excellent starting point for the investigation of the effects of radiations on cells.

Materials and Methods

White laboratory rats were starved overnight and sacrificed by a blow on the head. The liver was removed and placed in cold 0.85% KCl, where much of the blood was washed free. The liver was weighed and pressed through a bronze screen to remove connective tissue. The resulting mash was then suspended in 50 ml. of cold 8.5% sucrose containing 0.005 *M* disodium versenate and homogenized in a glass homogenizer with a "Teflon" pestle driven by a cone-drive stirring motor. The mitochondria were then separated from the rest of the homogenate by the method of differential centrifugation (Schneider, 1948). The mitochondrial fraction alone was kept.

In the studies of the effect of gamma radiation on the activity of cytochrome oxidase, the mitochondria were suspended in 2.5 ml. of sucrose-versenate per gram of original liver. For irradiation, a sample of the suspension was diluted 1 in 20 with distilled water. Five milliliters of the dilute preparation were put in a glass cup and irradiated in the beam of a 440-curie Co^{60} source. The radiation was filtered by a half-inch of lucite to remove beta radiation; the intensity of the radiation was 1000 r per minute. Controls were kept in a sheltered alcove outside the radiation room under the same conditions as the radiated material. The Warburg assays were run with the diluted preparations.

Treatment of the preparation for the determination of the effect of gamma radiation on phosphorylation differed from the above in some respects. The initial preparation was made by suspending the mitochondria in one ml. of sucrose-versenate per gram of liver. This suspension was then diluted 1 in 20 and radiated in a 25-ml. "Lusteroid" centrifuge tube. The controls were treated in the same manner, with the exception of the exposure to the radiation. After the radiation, control and treated suspensions were centrifuged, and the mitochondria re-suspended in one ml. This final suspension was assayed for phosphorylation.

The cytochrome oxidase activity was estimated manometrically by the method of Hogeboom, Claude and Hotchkiss (1946). The main compartment of each vessel contained: 0.35 ml. of mitochondrial suspension, 0.1 ml. Sorenson phosphate buffer (pH 7.4), 1 ml 1.3×10^{-4} *M* cytochrome-*c* (Sigma, horse-heart) in 0.85% NaCl, and 0.15 ml. 0.005 *M* $AlCl_3$. The center well contained 0.1 ml. 5 N KOH, and the side arm held the reducing agent, 0.15 ml. 0.228 *M* sodium ascorbate.

Estimation of phosphorylation was concluded by a modification of the method of Maley and Lardy (1954), using succinate as the substrate. The main compartment of the vessel contained: 0.3 ml. (30 μM) phosphate buffer (pH 7.4), 0.3 ml. 0.1 *M* sodium succinate, 0.8 ml. 8.5% sucrose, 0.1 ml. (0.3 μM) cytochrome-*c*, 0.3 ml. (6 μM) ATP (Schwartz, neutral), 0.1 ml. (30 μM) $MgSO_4$, 0.1 ml. (40 μM) KF, and 0.5 ml. of the mitochondrial suspension. The center well contained

0.1 ml. 5 N KOH, and the side arm held 0.5 ml. (20 mg.) of hexokinase (Pabst). To assure that the final pH of the reaction would be 7.0 or higher, the pH of some of the more acid reactants was adjusted with NaOH before addition to the flasks. Failure to do this results in lowered oxygen uptake and lowered phosphorylation. Readings of the oxygen uptake were taken for 30 minutes, after which time the reactions were stopped with TCA and the phosphate determined by the Lowry-Lopez method as presented by Glick (1949).

Assays of oxidase activity were made at 38° C.; assays of phosphorylation were made at 25° C. Assays of oxygen uptake were made in triplicate; assays of phosphorylation were made in duplicate. All experiments were repeated at least three times.

Results

Table I presents the data obtained from radiation of mitochondrial preparations of differing age. The preparation labeled "Day 1" was radiated on the same day

Table I

Inactivation of cytochrome oxidase by gamma radiation

Dose r	Day 1		Day 2		Day 3	
	No. runs	Per cent inactivation	No. runs	Per cent inactivation	No. runs	Per cent inactivation
2,500	2	1.8±4.1	6	6.4±3.8	6	5.7±6.0
4,000	8	4.8±2.4	18	18.9±2.9	4	8.7±2.2
5,000	13	9.4±2.9	18	8.8±2.1	12	10.2±4.4
10,000	6	10.5±2.2	10	6.5±1.8	9	10.5±2.6
12,500	8	16.7±2.4	8	25.7±4.0	10	27.1±3.2
15,000	14	17.4±2.9	17	26.7±2.0	7	26.5±1.6
20,000	19	29.9±2.4	16	29.7±2.3	16	26.7±2.1
30,000	5	41.3±3.5	11	34.6±2.5	6	31.0±6.5
40,000	6	49.1±2.8	10	42.8±4.5	12	34.1±3.3

that it was extracted; the preparation labeled "Day 2" was radiated on the following day; etc. These data show that cytochrome oxidase is extremely resistant to gamma radiation. This is in accord with the earlier studies of Barron *et al.* (1949). Nevertheless, although the data are extremely erratic below 10,000 r, some effect is achieved with doses as low as 2500 r. There is little indication that a maximum has been reached at 40,000 r. It is necessary to comment on the variability shown by the data. The figures are averages of several runs done with different rats. We have found that preparations from different rats give different results. In the case of the data obtained with 4000 r, Day 2, the inactivation varied from 12.1 per cent to 36.5 per cent. Whether this intrinsic variability is the result of differences in age, sex, or physiological condition, we are unable to judge at this time. In addition, there is always the problem of variation in the concentration of the preparations. All dilutions are made from suspensions made up as 2.5 ml. per gram of liver extracted. There is no reason to suppose that the number of mitochondria will be the same in each case. Until studies are done in

TABLE II

The effect of aging on the inactivation of cytochrome oxidase

Dose r	Morning		Afternoon	
	No. runs	Per cent inactivation	No. runs	Per cent inactivation
5,000	4	0.0±2.0	4	16.9±2.0
15,000	4	7.4±1.5	4	21.9±2.4
20,000	4	24.5±4.5	4	39.6±1.8

which the number of mitochondria in each sample are the same, no conclusions can be drawn about the variability between rats.

The data in Table I indicate that the preparations become more sensitive to low doses of radiation with time. On the first day, a dose of 2500 r produces little or no inactivation; on the second day, it produces about 6 per cent. This is shown in a much more striking manner in Table II. In this case the data from some runs done on the first day are broken down into those done in the morning and those done in the afternoon. In all cases, a preparation was radiated in the morning and afternoon, the only difference being the age. It is evident that when the mitochondria are first extracted they are much more resistant to radiation, particularly at the lower doses. At high doses, Table I indicates that there is a progressive decrease in sensitivity with age. Intermediate doses are erratic. As a result of

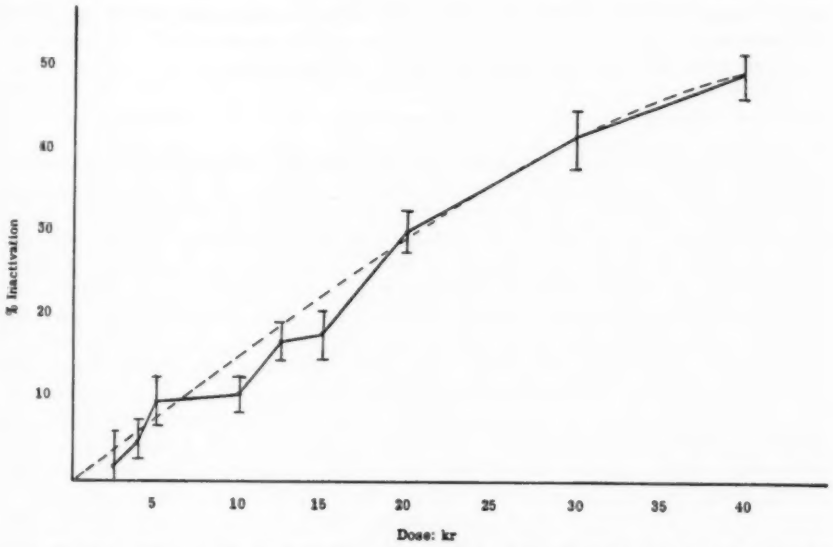

FIGURE 1. Inactivation of cytochrome oxidase by gamma radiation. Points represent means, with standard errors shown as limits. Preparation radiated the same day it was extracted. The dotted line represents the best fit of a regular curve.

this aging effect, the inactivation curve shown in Figure 1 was constructed on the basis of the first-day results only.

The data presented in Table III show that the oxidative phosphorylation mechanism is far more sensitive to radiation than cytochrome oxidase. All runs were done with a fresh preparation. There is no appreciable difference in the age of the preparations. In this case, each run represents a different rat, so that the variability results from this alone. It can be seen that there is little effect of the radiation upon the oxygen uptake in any case. The stimulation of phosphorylation by 2500 r is slightly greater than would be expected on the basis of increased

TABLE III

Inactivation of phosphorylation by gamma radiation

Dose	Phosphate uptake:		Per cent decrease	No. treated	O_2 uptake, % decrease
	Controls	Treated			
2,500	6.6±1.0	6.8±0.64	−3.0	10	−9.3
5,000	8.0±1.3	7.5±1.1	6.2	10	7.9
10,000	6.7±1.1	5.1±0.62	23.8	10	6.5
15,000	9.1±1.4	6.4±0.47	29.6	11	5.3
20,000	9.2±2.5	4.4±1.3	52.2	3	7.5
30,000	11.8±2.6	4.9±1.0	58.5	5	5.4
40,000	6.2±0.93	1.7±0.36	72.6	6	8.3

oxygen uptake, when calculations are made using a theoretical P:O ratio of 2 for succinate.

In all tables the data are shown as means ± standard error.

DISCUSSION

The data presented in this paper indicate that the inactivation of cytochrome oxidase by gamma radiation follows an irregular course. In all cases, there is a plateau reached at 5000 r to 10,000 r, followed by a sharp increase between 10,000 r and 12,500 r. The plateau is not lost upon aging (if anything, it is intensified); nor is it absent in fresh preparations. Table II indicates that in very fresh preparations there is a sharp increase between 15,000 and 20,000 r. It appears that the age of the preparation merely increases the dose necessary to cause the "jump." This observation requires special consideration. In the oxidation of cytochrome-*c* by ionizing radiation, it has been shown that the effects of radiation doses below 10,000 r are completely reversible (Barron, 1954). It is possible that part of the curve (Fig. 1) between 10,000 r and 15,000 r represents a shift from indirect to direct effects upon the particulate. Since we are radiating the entire particulate system, we can expect that the terminal oxidase of this system is constantly being reduced by substrate (either the supplied cytochrome-*c* or internal metabolites). This continual reduction may protect the cytochrome oxidase from the effects of the radiation, either during the time of radiation, when it must draw upon its internal supplies, or during the assay procedure, when it is supplied with a reducing agent which may effect post-irradiation recovery. Support for this idea comes from the aging effect shown in Table II. If the oxidase were being pro-

tected by materials within the mitochondrion at the time of radiation, we would expect that aging the preparation would lead to depletion of the internal stores with a consequent lowering of the protective effect. This is what is observed. At higher doses this protective effect is apparently negligible compared to the dose administered. Thus the effect of the radiation is reduced with age as might be expected from the protective effect of particulates whose oxidase has been inactivated by causes other than radiation.

It may be argued that the protection is merely the result of the various solute molecules which are not removed by washing during the extraction. It is difficult to decide whether there is a specific type of protection resulting from the generation of "reducing power" from the substrate, or whether there is a non-specific protective effect of additional substrate (Dale, 1942). It is clear that the effective substance is lost during aging; therefore, it seems sure that the protective substance must be within the particulate to be effective. If the substance were merely lost from the mitochondrion, it should be in the suspending medium which is diluted before radiation. The same concentration of substance would be present in either case. Such considerations raise the question of whether the effects of the radiation are independent of the concentration of the mitochondria. It might be suspected that only those ionizations produced within a mitochondrion would affect the enzymes within the structure. The particulate is sufficiently large to justify such an assumption. However, this does not prove to be the case. It was first established that preparations diluted in sucrose were less easily inactivated than those diluted in distilled water. This suggests that the ionization products of water are acting on the mitochondrion and that the sucrose is acting as a non-specific protective agent. Unfortunately this is not a clear test. The sucrose might be causing a change in the osmotic condition of the particulate, so that the protective substance (normally lost by aging) is more concentrated within the mitochondrion, or is not lost as readily. However, the data presented in Table III indicate that there is a dependance upon dilution. It can be seen that in a preparation 2.5 times as concentrated as that used to obtain the data in Table I, 40,000 r produce 8.3 per cent inactivation of oxygen uptake, whereas they produce 49.1 per cent in the dilute preparation. Clearly, 49.1 per cent is much greater than 2.5 times 8.3 per cent. Studies of intermediate dilutions bear this out. It is necessary to conclude that the effect of radiation upon the particulates is indirect in the sense that any solute molecule outside the mitochondrion will exert some protective effect upon the enzymes which are internal. Therefore, we must further conclude that the effect of the protective substance which is lost upon aging must be the result of some action it has *within* the mitochondrion, and that the loss which occurs with age probably results from the destruction of the substance by the particulates. As pointed out above, it is impossible to be sure of the mode of action of this protective agent, but the generation of materials which keep the enzymes in a reduced condition seems a likely mechanism.

A second explanation of the sudden "jump" in inactivation is that at doses over 10,000 r the particulate structure is undergoing severe change. This might result in the freeing of enzyme molecules, with consequent dilution of protective substances or in the loss of function of one part of the system with its release from another part. This seems a very unlikely mechanism. The fact that cytochrome oxidase can be freed from the rest of the particulate and still retain its activity

(Eichel *et al.* 1950) makes such an hypothesis difficult to maintain. Examination of the preparations by phase contrast did not show any radical changes; however, any alterations of ultrastructure could only be detected by other methods.

The dilution effect deserves additional comment. These experiments were originally designed in the hope that we would be able to approximate the biological condition. Radiation studies with dilute solutions of pure enzymes are informative with regard to radiation problems, but somewhat off the point for biological problems. No cellular system exists which is a single molecular species. Attempts to show that enzyme damage is the cause of radiation death in cells have been relatively unsuccessful (LeMay, 1951). On the other hand, the cell is so complex that it may die from many different causes, and it is difficult to be sure that one is investigating the right system in any particular case. It seemed necessary to investigate the problem in a system which had biological characteristics but which was not quite so diffuse as a whole cell. The particulates appear to offer such a system. It is possible to extract a "package" of enzymes, each of which has a relationship to other enzymes in the "package." The "package" resembles a cell in many aspects, but it is much simpler in its total organization. Furthermore it seemed that it was of sufficient size that only those ionizations produced within the mitochondrion would have any effect on the internal enzymes. This would be of great importance to the radiobiologist since it would indicate that there are sub-cellular bodies which can be considered to be separate from the rest of the cell, with regard to radiation damage. The chromosomes are frequently considered to be bodies of this type. Unfortunately this does not seem to be the case. The data presented in this paper indicate that ionizations external to the mitochondrion may cause internal damage, in a system containing only particulates and distilled water. It would appear that, at the doses studied, the ionization products of water are capable of producing their effects over relatively great distances, or that the effects of these products on the surface of the mitochondrion are capable of reducing the activity of the enzymes which are internal. This is of special interest as it has been suggested that the major effect of radiation on cytochrome-*c* is produced by hydroxyl radicals alone (Barron, 1954). Knowledge of the exact position of the oxidase in the particulate would be necessary to any final conclusion about these effects. However, the difficulties encountered in the extraction of cytochrome oxidase (Eichel *et al.*, 1950) suggest that the enzyme is internally bound.

From the foregoing discussion, it seems evident that we cannot conclude that cytochrome oxidase is damaged to any great extent by radiation doses used in most biological studies. It is difficult to know the concentration of the particulates in any cell, but it seems that the final suspension used in the phosphorylation studies (one ml. per one gm. liver) would best approximate the natural condition in liver cells. At no time was radiation given to particulates at this dilution. The strongest preparation ever used (during radiation) was 20 times diluted. At this dilution (Table III) there is little inactivation at 40,000 r. It is interesting that the effect on the oxidase seems to be the same for a wide range of doses. It is possible that in any dilution some small fraction of the activity would be lost, but this seems to be an unlikely cause of cell death. It is necessary to note that many cells do not have the high concentration of particulates which liver has. In these cases the effective doses necessary to inactivate cytochrome oxidase might fall within the

limits of biological experimentation, even with the protective substance of the cytoplasm present. In highly organized forms, the failure of one part may result in the death of the whole, so that damage to this system might *in some cases* result in death. This is particularly true of the forms which require fantastic doses of radiation to induce lethal changes.

When one considers the data in Table III, it becomes apparent that the oxygen uptake may be a faulty criterion for estimate of the health of a cell. These data show quite clearly that the phosphorylation mechanism is much more sensitive than the oxidase. Here we have a case in which over 70% of the ability to conserve energy as organic phosphate is gone with no apparent effect on the oxygen uptake. Considering the vital role of this system in the life of the cell, it seems quite probable that disruption of vital processes would result from the loss of 25 per cent (or less) of the ability to phosphorylate. We do not know how prevalent this loss is in the whole system. In these studies only the uptake which resulted from the oxidation of succinic acid was measured. It is possible that the whole phosphorylation mechanism of the particulates is damaged. Studies to determine the extent of the damage and to determine whether phosphorylation is carried out by a single system for all substrates are now in progress. In any case, it is evident that the phosphorylation mechanism is subject to destruction by ionizing radiation and that inactivation is achieved in relatively concentrated preparations which are very fresh. Although such preparations must be high in the concentration of the protective substance found for cytochrome oxidase, there seems to be little protection of the phosphorylation mechanism. This may be an indication that the protection mechanism is specific for the electron transport system (if not for cytochrome oxidase itself) or that the phosphorylation mechanism is extremely sensitive to radiation.

These data suggest an explanation for several different phenomena which have radiation as their sole common element. The phenomena are: induced crossing-over, induced tumor formation, and the general protective effect exerted by reducing compounds of the cysteine type. It has been suggested that one basis for the changes in genetic crossing-over induced by radiation is the alteration in the availability of phosphate linkages within the chromosome (Yost and Benneyan, 1957). It is evident that alterations in the phosphate pool of the cell must result from the type of damage described in this paper. Indeed, it is to be expected that such changes will have drastic effects on the chromosome structure, as many studies have already indicated (Haas *et al.*, 1954). It is also possible that *some* radiation-induced tumors are the result of the uncoupling of the oxidative metabolism of the cell. This could result in a situation similar to that which Warburg has suggested several times (Warburg, 1956). In cases in which oxygen uptake alone is measured, there is no assurance that the phosphorylation mechanism is functioning. Lastly, the general effect of reducing agents may be more than the maintenance of vital sulfhydryl groups. It is possible that the actual utilization of these compounds as reducing agents in the general metabolism will result in the protection of many non-sulfhydryl systems of the cell.

Summary

Data are presented which indicate that the phosphorylation mechanism is much more sensitive to gamma radiation than cytochrome oxidase. It is suggested that

the utilization of substrates by enzymes within the particulate may protect these enzymes against ionizing radiation. Various consequences of these findings are discussed.

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